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VOLUME 2

GRAS MONOGRAPH SERIES

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prepared for
THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

JULY 29, 1974

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THE BIOLOGIC RELATIONSHIP BETWEEN COW'S, GOAT'S AND HUMAN CASEINS

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Clinical observations indicate the possibility that there is an immunologic relationship between human, cow's and goat's milks. It is a matter of common experience that eczema and other manifestations of hypersensitiveness in the nursing baby are frequently aggravated by the addition of cow's milk, and conversely that eczema in the milk-sensitized infant is unrelieved by reverting to human milk or by the substitution of goat's milk. The work of Fleischer [1] Bauer,[2] Wells [3] and von Versell [4] suggested that this interrelationship may reside in a biologic similarity of the caseins of the three milks. The establishment of this fact seemed to be of sufficient importance, because of its practical application, to warrant further investigation. With this end in view, we undertook a series of experiments in which we used purified cow's, human and goat's caseins. The results of this work form the basis of the present communication.

The caseins that were used in the experiments were prepared by the following method.

From the Department of Pediatrics, Cornell University Medical College and the New York Nursery and Child's Hospital, New York City; and from the Department of Pediatrics, Harvard Medical School and The Children's Hospital, Boston.

1. Fleischer, G. W.: Russk. Vrach **7** (pt. 2) :1638, 1908; abstr., Centralbl. f. Path. **20**:308, 1909.

2. Bauer, J.: Ueber den Artcharakter der Milcheiweisskörper, Klin. Wchnschr. **47**:830, 1910.

3. Wells, H. G.: Studies of the Chemistry of Anaphylaxis: III. Experiments with Isolated Proteins, Especially Those of the Hen's Egg, J. Infect. Dis. **20**:147, 1911.

4. von Versell, Arnold: Ueber das serologische Verhalten von Milch und Milcheiweisskorpern in frischem und gekochtem Zustand, Ztschr. f. Immunitätsforsch. u. exper. Therap. **24**:267, 1915-1916.

METHOD OF PREPARATION

Cow's Casein.—Fat-free raw milk was used entirely. Early in the work, the casein was precipitated with tenth-normal acetic acid, following the determination of the maximum precipitation point by repeated titrations of the milk. Later, it was prepared by the method of Van Slyke and Baker.[5] This permits of the removal of the insoluble calcium caseinate and calcium phosphate at a point just below the iso-electric point, thereby rendering the casein much more soluble.[6] One per cent solutions of this product were obtained by dissolving 0.1 Gm. of casein in 9 cc. of physiologic solution of sodium chloride and 1 cc. of tenth-normal solution of sodium hydroxide.

Human Casein.—The raw milk was centrifugated to remove the fat. It was then dialyzed against distilled water to remove the buffer salts. Early in the work, it was precipitated with twentieth-normal acetic acid. Later, it was found that by using a partially buffered solution of twentieth-normal lactic acid and fortieth-normal sodium hydroxide in the apparatus described by Van Slyke and Baker for the preparation of cow's casein[5] a much larger yield of a readily soluble casein could be obtained.[6] Two per cent solutions of the product were obtained by dissolving 0.2 Gm. of casein in 9.2 cc. of physiologic solution of sodium chloride and 0.8 cc. of tenth-normal solution of sodium hydroxide.

Goat's Casein.—The fat of raw milk was removed by centrifugation. The casein was precipitated by the addition of tenth-normal acetic acid. It was then freed from electrolytes by washing with water and was dried with alcohol and ether. One per cent solutions of the product were obtained by dissolving 0.1 Gm. of casein in 9 cc. of physiologic solution of sodium chloride and 1 cc. of tenth-normal solution of sodium hydroxide.

ACTIVE SENSITIZATION

This phase of the work consisted of the active sensitization of guinea-pigs with one of the caseins and subsequent attempts to produce anaphylaxis with one of the other caseins.

Negative controls in each experiment consisted of normal animals that were given intravenous injections of the second casein. In none of these animals were the injections attended by any evidences of shock or untoward disturbances that simulated anaphylaxis. Positive controls consisted of animals that were sensitized by the intraperitoneal route to the first casein and were subsequently given intravenous injections of the same casein. In all of these, the second injection resulted in violent anaphylaxis.

EXPERIMENTS

GROUP 1.—Forty-six animals were sensitized to cow's casein by the intraperitoneal route. From seventeen to twenty-one days later they were given human casein intravenously. The results are summarized in table 1.

5. Van Slyke, L. L., and Baker, John C.: The Preparation of Pure Casein, J. Biol. Chem. **52**:127, 1918.

6. Casein prepared by this method was used entirely in the experiments on passive sensitization and precipitin tests and to a large extent in those on active sensitization.

Of the forty-six animals, thirty-two presented evidence of an immunologic relationship between the two caseins.

It seemed of interest to determine whether or not the animals that were anaphylactic but recovered after the injection of human casein were desensitized to the original antigen, cow's casein. Accordingly, thirteen animals in this group were given a second injection of cow's casein within a few hours. All of these animals died in typical shock. This seems to indicate that complete desensitization to the original antigen did not occur. These results are similar to those obtained by Wells [8] in his experiments on the relationship between cow's and goat's caseins, in which he found that desensitization to the original and sensitizing antigen did

TABLE 1.—*Guinea-Pigs Sensitized to Cow's Casein Intraperitoneally; from Seventeen to Twenty-One Days Later, Human Casein Was Given Intravenously*

a. Animals died in anaphylactic shock*	8
b. Animals anaphylactic but recovered	24
c. Equivocal signs	6
d. No evidence of anaphylaxis	8
Total	46

* In the preparation of the data shown in this and the following tables, the criteria of anaphylaxis in all of these animals were convulsions, severe respiratory difficulty, involuntary twitchings and frequently transitory paralyses of the extremities, sneezing, ruffling of the coat, loss of sphincter control and bucking.

TABLE 2.—*Guinea-Pigs Sensitized to Human Casein Intraperitoneally; from Seventeen to Twenty-One Days Later, Cow's Casein was Given Intravenously*

a. Animals died in anaphylactic shock	2
b. Animals anaphylactic but recovered	14
c. Equivocal signs	5
d. No evidence of anaphylaxis	16
Total	37

not occur after recovery from severe anaphylaxis that was induced by the injection of the opposing casein. The significance of this we shall discuss later.

GROUP 2 (Reverse of Group 1).—Thirty-seven animals were sensitized to human casein by the intraperitoneal route. From seventeen to twenty-one days later, they were given cow's casein intravenously. The results are summarized in table 2.

Of the thirty-seven animals, sixteen presented evidence of an interreaction between the caseins.

It is of interest to note that of the twelve animals in the group that were anaphylactic but recovered after the injection of cow's casein and were subsequently given injections of human casein ten died in a state of anaphylactic shock. These findings are analogous to those obtained in the animals in group 1 and point to a lack of complete desensitization to the original antigen.

Confirmatory evidence for the findings in groups 1 and 2 was sought by means of the Schultz-Dale phenomenon. Accordingly, six female guinea-pigs were actively sensitized to cow's casein. From seventeen to twenty-one days later, a uterine horn was removed and suspended in Ringer's solution. A solution of human casein was then added to the bath. In three of the experiments there was

a sharp response to the introduction of the human casein. On addition of the original antigen, cow's casein, however, there was a second and greater reaction on the part of the uterine strip (chart 1). Similarly, in two of six experiments on animals that were previously sensitized to human casein, there was an immediate response of the uterine strip when a solution of cow's casein was added, and a second and complete contraction when the original antigen, human casein, was subsequently introduced (chart 2). These results are in keeping with those obtained in the preceding experiments in active sensitization.

GROUP 3.—Twelve animals were sensitized to goat's casein by the intraperitoneal route. From seventeen to twenty-one days later, they were given human casein intravenously. The results are summarized in table 3.

TABLE 3.—*Guinea-Pigs Sensitized to Goat's Casein Intraperitoneally; from Seventeen to Twenty-One Days Later, Human Casein was Given Intravenously*

a. Animals died in anaphylactic shock	1
b. Animals anaphylactic but recovered	8
c. Equivocal signs	0
d. No evidence of anaphylaxis	3
Total	12

TABLE 4.—*Guinea-Pigs Sensitized to Human Casein Intraperitoneally; from Seventeen to Twenty-One Days Later, Goat's Casein Was Given Intravenously*

a. Animals died in shock	2
b. Animals anaphylactic but recovered	2
c. Equivocal signs	0
d. No evidence of anaphylaxis	0
Total	4

In nine of the twelve animals, there was evidence of an interreaction between the two caseins.

It is of interest that two animals in the group that were anaphylactic but recovered after the injection of human casein died in anaphylactic shock when subsequently given injections of the original antigen.

GROUP 4 (Reverse of Group 3).—Four animals were sensitized to human casein by the intraperitoneal route. From seventeen to twenty-one days later they were given goat's casein intravenously. The results are summarized in table 4.

In all of the animals comprising this group, there was an interrelationship between the two caseins. Both animals that were anaphylactic but recovered after the injection of goat's casein died in anaphylactic shock when subsequently given injections of the sensitizing antigen.

The findings in groups 3 and 4 are comparable to the results obtained in groups 1 and 2 and point to the same similarity between human and goat's caseins as exists between cow's and human caseins.

GROUP 5.—Eight animals were sensitized to cow's casein by the intraperitoneal route. From seventeen to twenty-one days later they were given goat's casein intravenously. The results are summarized in table 5.

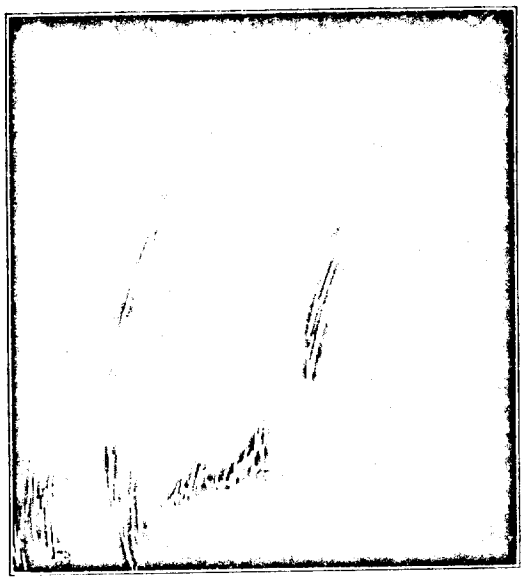

Chart 1.—Results of the Dale test, showing response of uterine muscle to cow (*CC*) and human (*HC*) caseins in guinea-pigs originally sensitized to cow's casein.

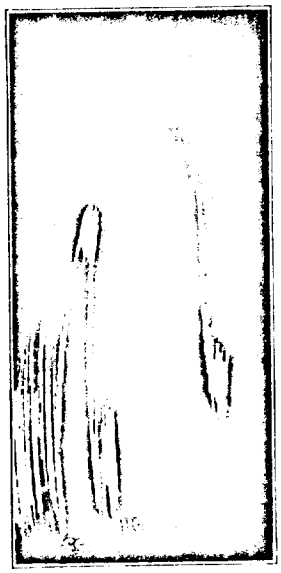

Chart 2.—Results of the Dale test, showing response of uterine muscle to cow (*CC*) and human (*HC*) caseins in guinea-pigs sensitized to human casein.

In five of the eight animals there was an interreaction between the two caseins.

GROUP 6 (Reverse of Group 5).—Three animals were sensitized to goat's casein. Seventeen days later they were given cow's casein intravenously. The results are summarized in table 6.

In all of the animals in this group there was an interreaction between the two caseins.

The results of experiments in groups 5 and 6 are in keeping with those reported for the four preceding groups, and indicate a relationship between cow's and goat's caseins comparable to that found to exist between cow's and human, and human and goat's caseins.

TABLE 5.—*Guinea-Pigs Sensitized to Cow's Casein Intraperitoneally; from Seventeen to Twenty-One Days Later, Goat's Casein Was Given Intravenously*

a. Animals died in anaphylactic shock	3
b. Animals anaphylactic but recovered	2
c. Equivocal signs	0
d. No evidence of anaphylaxis	3
Total	8

TABLE 6.—*Guinea-Pigs Sensitized to Goat's Casein Intraperitoneally; from Seventeen to Twenty-One Days Later, Cow's Casein Was Given Intravenously*

a. Animals died in anaphylactic shock	0
b. Animals anaphylactic but recovered	3
c. Equivocal signs	0
d. No evidence of anaphylaxis	0
Total	3

PRECIPITIN TESTS

In this phase of the work, we endeavored to determine the ability of known anti-cow's casein and anti-human casein rabbit serums to precipitate the opposite antigen.

Anti-cow's casein serum was obtained by actively immunizing rabbits by the intravenous injection of 5 cc. of a 1 per cent solution of cow's casein every second day for a period of two weeks. Only serums with titers for cow's casein of at least 1:500 were used in the tests. It was found early in the work that both cow's casein and human casein were only weakly precipitinogenic and that their anti-serums rarely gave a precipitin titer of greater than 1:200. Later we were able to increase the titer to 1:500 by using rabbits that previously had been immunized to another and entirely unrelated protein, such as eggwhite, as recommended by Parker[7] for the preparation of pollen extract precipitins. Anti-human casein serum was obtained in a similar manner, with 5 cc. of a 2 per cent

7. Parker, J. T.: Production of Precipitins for Ragweed Pollen, J.Immunol. 9:515 (Nov.) 1924.

solution of human casein as the antigen. Serums yielding a titer of at least 1:500 were used throughout.

The tests for precipitin were made as follows: A 1 per cent solution of cow's casein and a 2 per cent solution of human casein were diluted to 1:20, 1:50, 1:100, 1:250, 1:500 and 1:1,000. To each of these dilutions were added 0.1 and 0.2 cc. of the opposite immune serum. The tests were read after one hour's incubation at 37.5 C., and again after the solution had stood on ice for eighteen hours. Controls of normal rabbit's and human serum were used throughout. Dilutions of the casein solution without the addition of serum and immune serum diluted with physiologic solution of sodium chloride also served as controls. Only tests that showed distinct flocculation were recorded as positive. They may be summarized as follows: 1. Anti-cow's casein rabbit serum, which gave a precipitin titer to cow's casein of 1:500, regularly precipitated human casein in a dilution of 1:300. 2. Anti-human casein rabbit serum, which gave a precipitin titer to human casein of 1:500, precipitated cow's casein in a dilution of 1:250.

In brief, the immune serums contained precipitins that reacted to both caseins, although the titer was considerably weaker for the opposite antigen. These findings are comparable to those obtained in the experiments in active sensitization.

TABLE 7.—*Guinea-Pigs Passively Sensitized to Cow's Casein by the Intraperitoneal Injection of Anti-Cow's Casein Rabbit Serum; Twenty Hours Later, Human Casein Was Given Intravenously*

a. Animals died in anaphylactic shock	0
b. Animals anaphylactic but recovered	15
c. Equivocal signs	9
d. No evidence of anaphylaxis	3
Total	27

PASSIVE SENSITIZATION

In these experiments we passively sensitized guinea-pigs with known anti-cow's casein and anti-human casein rabbit serums and attempted subsequently to produce anaphylaxis with the opposite antigen.

GROUP A.—Twenty-seven guinea-pigs were passively sensitized by the intraperitoneal injection of from 1 to 3 cc. of anti-cow's casein serum, prepared by the method described in the report of the precipitin tests. From eighteen to twenty-one hours later, the animals were given 1 cc. of a 2 per cent solution of human casein intravenously. The results are summarized in table 7.

Of the twenty-seven animals, fifteen showed evidence of an interreaction between the caseins. Of these, six were within four hours given injections of the original antigen, cow's casein. Five of these six animals died in a typical state of shock.

Positive controls consisted of a group of six guinea-pigs that were passively sensitized to cow's casein in the same manner as the test animals and later given the original antigen, cow's casein, intravenously. All of these controls died in a state of anaphylactic shock. Negative controls consisted of a second group of five normal guinea-pigs, not previously sensitized with immune serum, which were given human casein intravenously. The results in these animals were uniformly negative.

GROUP B.—Twenty guinea-pigs were passively sensitized by the intraperitoneal injection of from 1 to 3 cc. of anti-human casein serum obtained by the method previously described. From eighteen to twenty-one hours later, they were given 1 cc. of a 1 per cent solution of cow's casein intravenously. The results are summarized in table 8.

Of the twenty animals, nine presented evidence of an interreaction between the two caseins. Eight of the animals with positive reactions were subsequently given a second injection of human casein, and, of these, seven died in typical shock.

Four animals, passively sensitized in the same manner as the test animals, and subsequently given human casein intravenously died in a state of anaphylactic shock. Of four normal guinea-pigs that did not receive immune serum but that were given cow's casein intravenously, none presented any untoward signs. These two groups served as positive and negative controls, respectively.

The results of these experiments in passive sensitization are strikingly similar to those obtained in the work on active sensitization. In both there is strong presumptive evidence of an interreaction between cow's and human caseins. Also, complete desensitization did not occur following the induction of anaphylaxis.

TABLE 8.—*Guinea-Pigs Passively Sensitized to Human Casein by the Intraperitoneal Injection of Anti-Human Casein Rabbit Serum; Twenty Hours Later, Cow's Casein Was Given Intravenously*

a. Animals died in anaphylactic shock	0
b. Animals anaphylactic but recovered	9
c. Equivocal signs	8
d. No signs of anaphylaxis	3
Total	20

COMMENT

These experiments indicate a close immunologic relationship between the caseins of cow's, goat's and human milks. It would appear, therefore, as previously suggested by Versell,[4] that casein occupies a position analogous to lens and testicular proteins, in that it is not species-specific but possesses characteristics that are common to a number of different species.

The results that we obtained seem to point to slight chemical differences in the three caseins. Evidence of this is the failure of one casein completely to desensitize the animal against the sensitizing casein. This could be explained by the assumption that human, cow's and goat's casein each contain two distinct antigenic fractions. One of these is species-specific and sensitizes only to itself. The other antigenic factor is common to the three species. This assumption would explain at the same time the antigenic relationship of the different varieties of casein and also their specificity. A possible analogy to these variations is found in the experiments of Dochez and Avery,[8] Zinsser and Parker[9]

8. Dochez, A. R., and Avery, O. T.: The Elaboration of Specific Soluble Substance by Pneumococcus During Growth, J. Exper. Med. **26**:477, 1917.

9. Zinsser, H., and Parker, J. R.: Further Studies on Bacterial Hypersusceptibility: II., J. Exper. Med. **37**:275, 1923.

and Heidelberger and Avery [10] on the type-specific substance in the body fluids of patients with pneumonia, in which it was shown that antigenic substances derived from such closely allied organisms as the various types of pneumococci possess certain distinct chemical and biologic characteristics that clearly differentiate the types.

It seems to us that these findings may be of practical as well as of academic importance. Clinical experience has shown that a certain number of "milk hypersensitive" infants are markedly benefited when a change from cow's milk to human or goat's milk is made. In some of these infants, skin tests have revealed hypersensitiveness to lactalbumin of cow's milk, and it is reasonable to suppose that many more would react similarly if they were studied carefully. In view of the biologic dissimilarity between the whey proteins of the various milks, it is readily understood why such substitution measures are successful in this group of children. Unfortunately, however, this procedure is either only partially successful or entirely unsuccessful in a large number of infants who manifest allergic symptoms when fed cow's milk. It is possible that these failures may be due to sensitization to cow's milk casein rather than to lactalbumin. In this event, the substitution of human milk or of goat's milk for cow's milk would merely constitute a replacement by another biologically similar problem.

SUMMARY

1. Experiments in active sensitization revealed that cow's, human and goat's caseins sensitize against each other.

2. Anti-cow's casein serums gave positive precipitin reactions to human casein, and anti-human casein serums reacted positively with solutions of cow's casein.

3. Animals that were passively sensitized to cow's casein exhibited evidences of anaphylaxis when given human casein intravenously. The converse was equally true.

4. The results that were obtained seem to indicate a close biologic relationship between cow's, human and goat's caseins. It is possible that this similarity may explain certain difficulties that are encountered in the treatment of allergic states by the substitution of one milk for another.

161 West Sixty-First Street, New York City.

300 Longwood Avenue, Boston.

10. Heidelberger, M., and Avery, O. T.: The Soluble Specific Substances of Pneumococcus, J. Exper. Med. **38**:73, 1923. Avery, O. T., and Heidelberger, M.: Immunologic Relationships of Cell Constituents of Pneumococcus, ibid. **38**:81, 1923.

J. Nutr. 93(4): 429-437, 1967

Lethal Amounts of Casein, Casein Salts and Hydrolyzed Casein Given Orally to Albino Rats [1]

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ABSTRACT The lethal dose of casein given as an aqueous suspension intragastrically to albino rats was estimated to be well over 1000 g/kg administered over a period of 2 weeks but could not be definitely established because deaths were due in part to distilled water in the suspension. The LD_{50} of the water-soluble sodium and calcium salts of casein was estimated to be some 400–500 g/kg given over a 5-day period, the intoxication being due mainly to salt effects. The $LD_{50} \pm SE$ of pancreatin-hydrolyzed casein was found to be 26.0 ± 1.6 g/kg, death occurred at 2 to 4 hours and was due to a violent gastroenteritis, blood and tissue dehydration, widespread capillary-venous congestion, coma and respiratory failure. Survivors of the latter group recovered clinically in 2 to 3 days but some changes in organ weights were significantly abnormal at 2 weeks and even at 1 month. The results indicate it is almost impossible to administer lethal amounts of casein orally to albino rats but that toxic effects can be produced by water-soluble salts of casein and particularly by the amino acids and polypeptides of hydrolyzed casein.

During the course of studies on the toxicity of drugs given to adult albino rats fed various purified vitamin-deficient diets, evidence was obtained which suggested that toxicity may be associated with the presence of large amounts of certain foods in the diets (1). As a corollary it should be possible to demonstrate toxic and lethal doses of such foods. Certain preparations of casein were found to produce toxicity and death when given orally in sufficient amounts to albino rats. The amount required to produce death was far in excess of that likely to be fed or eaten except in the instance of hydrolyzed casein.

Hydrolyzed casein is produced in the digestion of casein with, for example, pancreatin (2). It is present in certain proprietary infant food formulas (3) and is used in the therapy of babies with special feeding problems (4) such as allergenic sensitivity to intact proteins, pancreatic deficiencies and tube feeding (it is water-soluble). The results of the present study suggest that if casein hydrolysate were fed to infants in amounts somewhat greater than the recommended 5 g/kg per day (4), it might produce toxic signs which could resemble the disease being treated. Rats may eat some 15 to 30 g/kg per day of casein (1) and these amounts of casein hydrolysate can produce lethal reactions if given to rats at one administration. The results of the present study, therefore, have important implications in the fields of animal and human nutrition.

MATERIALS AND METHODS

Techniques. Seven preparations of casein were given by intragastric cannula to overnight-starved, young, male, albino rats [2] weighing 150 to 200 g, in increasing amounts until death occurred. The animals were housed in metabolism cages, one rat per cage, and were offered laboratory ration [3] and water ad libitum. Clinical signs of toxicity were noted daily (or at shorter intervals if indicated) until the syndrome had subsided. At intervals of 24 hours measurements were made of body weight in grams, food consumption in grams per kilogram of body weight per day, water intake in milliliters per kilogram per day, colonic temperature, urinary volume in milliliters per kilogram per day, urinary

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[1] This research was supported by a grant from the Medical Research Council of Canada.
[2] The animals were a Wistar strain obtained from Canadian Breeding Laboratories of St. Constant, Quebec, Canada.
[3] Purina Laboratory Chow Checkers, Ralston Purina Company, Limited, Woodstock, Ontario, Canada.

protein, and glucose output semi-quantitated in milligrams per kilogram per day, and urinary pH on a 24-hour sample. The last 3 measurements were made with the use of Ames Combistix [4] and colonic temperature was recorded by a Thermistemp Telethermometer [5] with the probe inserted into the descending colon. Other measurements were made as indicated and as noted below.

Premortem signs were recorded in detail when possible and following death the gross and microscopic appearance of the organs listed in table 1 were noted. Histopathologic studies were made of blocks of tissue fixed in Lillie's buffered formalin and sections were stained with hematoxylin-phloxine-saffron. In addition, the wet weight [6] and water content of the organs listed in table 1 were measured at death and in survivors at 2 weeks and at one month following administration of enzymatic casein hydrolysate.[7] Autopsies were performed within one hour of death to avoid the postmortem shifts in weight and water content described by Boyd and Knight (5). The contents of the lumen of the gastrointestinal organs were removed by a standardized washing and milking before weighing. The sample of muscle was the right half of the ventral abdominal wall muscle layer. The medulla oblongata was included in dissection of the brain. Water content was determined upon organs dried to constant weight at 95° in a forced-draft isotemp oven.[8] Aliquots from the dorsolumbar region of skin and from residual homogenized carcass were used for measuring water content.

Mean differences from controls were subjected to the *t* test for statistical significance (6). The $LD_{50} \pm SE$ was calculated by the linear regression method of Boyd (7).

Preparations of casein. Casein certified Fisher [9] was administered as a 30% (w/v) suspension in a 0.12% solution of ammonium hydroxide in distilled water as suggested from the data of Davies (8). Following pilot tests with lesser volumes, it was finally given warmed to body temperature in a volume of 100 ml/kg which volume appeared feasible from the report of Boyd et al. (9). This was repeated at hourly intervals to yield doses of 30, 60, 90, 120 and 150 g/kg with controls given the same volumes of 0.12% ammonium hydroxide solution, each dose of casein and of vehicle to 10 rats.

Following preliminary trials, high protein casein [10] and vitamin-free test casein [11] were administered in a dose of 50 ml/kg of a 15% (w/v) suspension at 5 successive hourly intervals each day (= 37.5 g/kg per day) for 3 days and then the dose was gradually increased to 9 administrations per day until death occurred or for 3 weeks, whichever occurred first, each preparation to 20 rats. Following similar pilot studies, sodium caseinate,[12] casein sodium and casein calcium [13] were each given as 50 ml/kg of a 15% (w/v) solution in distilled water at 10 successive intervals of 0.5 hours (= 75 g/kg per day) each day until the animals died or for one week whichever occurred first, each preparation to 10 rats.

Enzymatic casein hydrolysate was dissolved in distilled water at body temperature and administered in a volume of 100 ml/kg in doses of 10, 20, 22.5, 24, 25, 26.

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[4] Manufactured by the Ames Company of Canada Limited, Rexdale, Ontario, Canada.

[5] Purchased from the Fisher Scientific Company Limited, Don Mills, Ontario, Canada.

[6] Organs were weighed to 0.1 mg on a 1-911X Mettler semi-micro gramatic balance, except skin and residual carcass which were weighed on a Mettler K-5T precision balance, both balances purchased from Fisher Scientific Company Limited, Don Mills, Ontario Canada.

[7] Enzymatic casein hydrolysate is produced by treating casein with pancreatin which converts casein into its amino acids and peptides. General Biochemicals Chagrin Falls, Ohio, from whom it was purchased, state that it is edible and that the degree of hydrolysis is "acceptable to the American Medical Association." The preparation was found to be soluble to 60% (w/v) in distilled water at body temperature.

[8] See footnote 5.

[9] See footnote 5.

[10] High protein casein was obtained from General Biochemicals, Chagrin Falls, Ohio, who reported that it was produced by the controlled lactic acid fermentation of pure skim milk and contained 85% protein, 11% water, 1.9% ash, 1.5% milk fat and small amounts of thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cyanocobalamin, copper, iron, zinc and iodine.

[11] Vitamin-Free Test Casein was obtained from General Biochemicals, Chagrin Falls, Ohio, who reported that it is prepared by controlled multiple extractions with hot alcohol and vacuum drying and contains 89.0% protein, 8.0% water, 2.0% ash and 0.5% fat. Alcohol extraction removes some 75% (35 to 100) of the vitamins contained in high protein casein so that it is not "vitamin-free" but may be considered such for most biological purposes.

[12] Sodium caseinate was obtained from General Biochemicals, Chagrin Falls, Ohio, who reported that it contained 92.5–94.5% protein, 4.0% ash (1.3% sodium) and 1.5% fat. It was found soluble to 20% in water at body temperature.

[13] Casein sodium and casein calcium were obtained from Nutritional Biochemicals Corporation, Cleveland. Casein sodium was found soluble in water to 25% and casein calcium to 15% at body temperature.

27.5, 29, 30, 40, and 50 g/kg each dose to 16 to 20 rats with 31 controls given 100 ml/kg of distilled water only.

RESULTS

Casein. Suspensions of casein Fisher gradually coagulated into particles of various sizes on standing and also in the rat's stomach. A dose of 30 g/kg killed 30% of the rats by regurgitation and aspiration into the lungs where it produced death from aspiration asphyxia, usually within 30 minutes, or from gastric rupture with death at 6 to 14 hours later. The LD_{50} of ammonium hydroxide, given in solution to the controls, was found to be 0.45 g/kg; death followed convulsoins at 0.5 to 4 hours and was accompanied by a violent gastritis. The LD_{50} of ammonium hydroxide is similar to that reported previously in cats by Spector (10).

High protein and vitamin-free test casein suspensions produced 40% deaths from stomach rupture after administration of 37.5 g/kg (day 1). There were no further deaths until the eighth day when daily administrations had increased to seven, or 52.5 g/kg per day, and a total of 345 g/kg of casein had been given — at which point 50% of rats had died of gastric rupture. Twenty percent of the rats survived 9 administrations or 67.5 g/kg per day for one week following the fourteenth day when this daily dose was reached. The clinical signs in these 8 rats were inhibition of growth, anorexia, a marked diuresis due largely to daily administration of large volumes of water, proteinuria, aciduria, and listlessness. These signs were accentuated premortally in 3 rats that died apparently of protein intoxication and at least not from gastric rupture or from regurgitation asphyxia.

Ability to survive large amounts of "natural" casein (high protein casein, vitamin-free test casein, and casein Fisher) appeared to be related mainly to ability of the stomach to accommodate the necessary large bulk. In survivors there was some capillary-venous congestion of the lamina propria and submucosa of the stomach, small bowel, cecum and colon. There was some evidence of systemic damage. The liver was congested and occasionally there were scattered areas of necrosis in the lobules. There was mild-to-moderate capillary-venous congestion of the brain, lungs, heart and kidneys and atrophic changes in the myofibrils of skeletal muscle and in the thymus gland. These changes were accentuated in 3 rats that died apparently of casein poisoning. The total dose of casein given to the latter 3 rats was 140, 340 and 830 g/kg given over 4, 8 and 15 days, respectively. If calculation of the LD_{50} can be applied under these circumstances, casein would have an LD_{50} well over 1000 g/kg.

Casein salts. Of the 10 rats given sodium caseinate, 2 died of gastric rupture during the first 24 hours and 3 died on the fourth and fifth days with no gastric rupture and no evidence of regurgitation asphyxia. The latter 3 animals had received 75 g/kg per day of sodium caseinate (containing approximately 1 g/kg per day of sodium) or a total of 300 to 375 g/kg over the interval to death. The indicated "LD_{50}" would be of the order of 400 to 500 g/kg. Death was preceded by marked loss of body weight, marked anorexia, marked diuresis, marked proteinuria, alkalinuria, diarrhea, listlessness and a premortal hypothermia. The local inflammatory reaction in the gut was confined mostly to the cecum. There occurred hepatic, renal, cerebral, cardiac, pulmonary, testicular, adrenal, thymic and splenic capillary-venous congestion and fatty degeneration of the renal tubules. Animals that survived had very marked diuresis and an augmented water intake.

Results in animals given casein sodium were similar to those obtained from sodium caseinate. Of the 10 rats given casein calcium, 8 died of stomach rupture during the first and second days and the reaction of the 2 survivors was similar to that seen in rats which survived administration of sodium caseinate.

Casein hydrolysate. The $LD_{50} \pm SE$ of enzymatic casein hydrolysate was found to be 26.0 ± 1.6 g/kg, the maximal LD_0 was 23.5 and the minimal LD_{100} was 28.6 g/kg. The mean $\pm$ SD interval to death was 3.6 ± 1.4 hours excluding one delayed death at 24 hours and a second at 27 days. The interval to death was shorter the higher the dose of the casein hydrolysate.

Clinical signs of toxicity during the first hour were listlessness, cyanosis, and diarrheic bowel movements which appeared

to consist mostly of casein hydrolysate. Hemoconcentration developed rapidly as shown in figure 1 and was greater the higher the dose of the casein hydrolysate as shown in figure 2. Death was due to respiratory failure in a cyanotic coma. At autopsy, most organs were found to have lost weight, as shown in table 1, due to loss of water, as shown in table 2. On gross observation, an intense congestion of the brain, hemorrhagic inflammation of the gut and a dark-colored liver were observed. Microscopic examination confirmed the gross pathology and disclosed vascular congestion in many other organs as indicated in table 3 and, in the delayed death, degenerative changes in the kidneys and lungs. Blood clots, noted in table 3, were due either to antemortem clotting or to accelerated postmortem clotting or to both, since they were not seen in controls given no casein enzymatic hydrolysate.

Survivors at 24 hours had lost body weight and exhibited anorexia, hyperdipsia, mild fever, diuresis, aciduria and proteinuria but appeared grossly normal, as indicated by results summarized in table 4. The data in table 4 are expressed as percentage of change from controls given 100 ml/kg of distilled water which in itself produced a moderate diuresis, alkalinuria, glycosuria and proteinuria. By the third day, clinical parameters were returning toward normal but not all organ weights and water

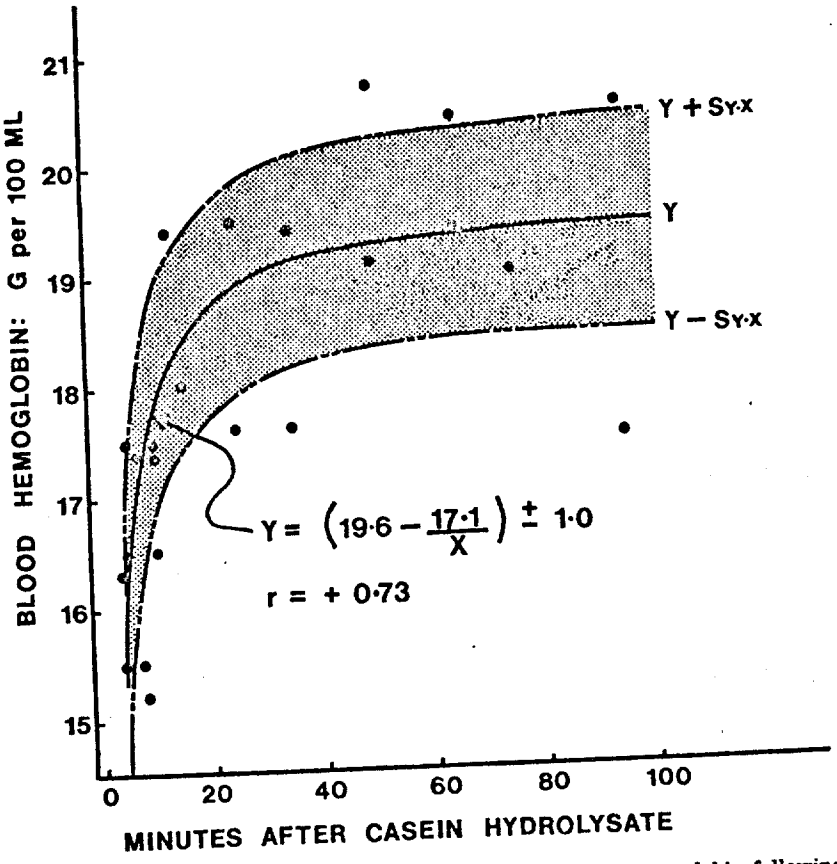

Fig. 1 The regression, on time in minutes, of values for blood hemoglobin following oral administration of casein enzymatic hydrolysate in a dose of 26 g/kg body weight.

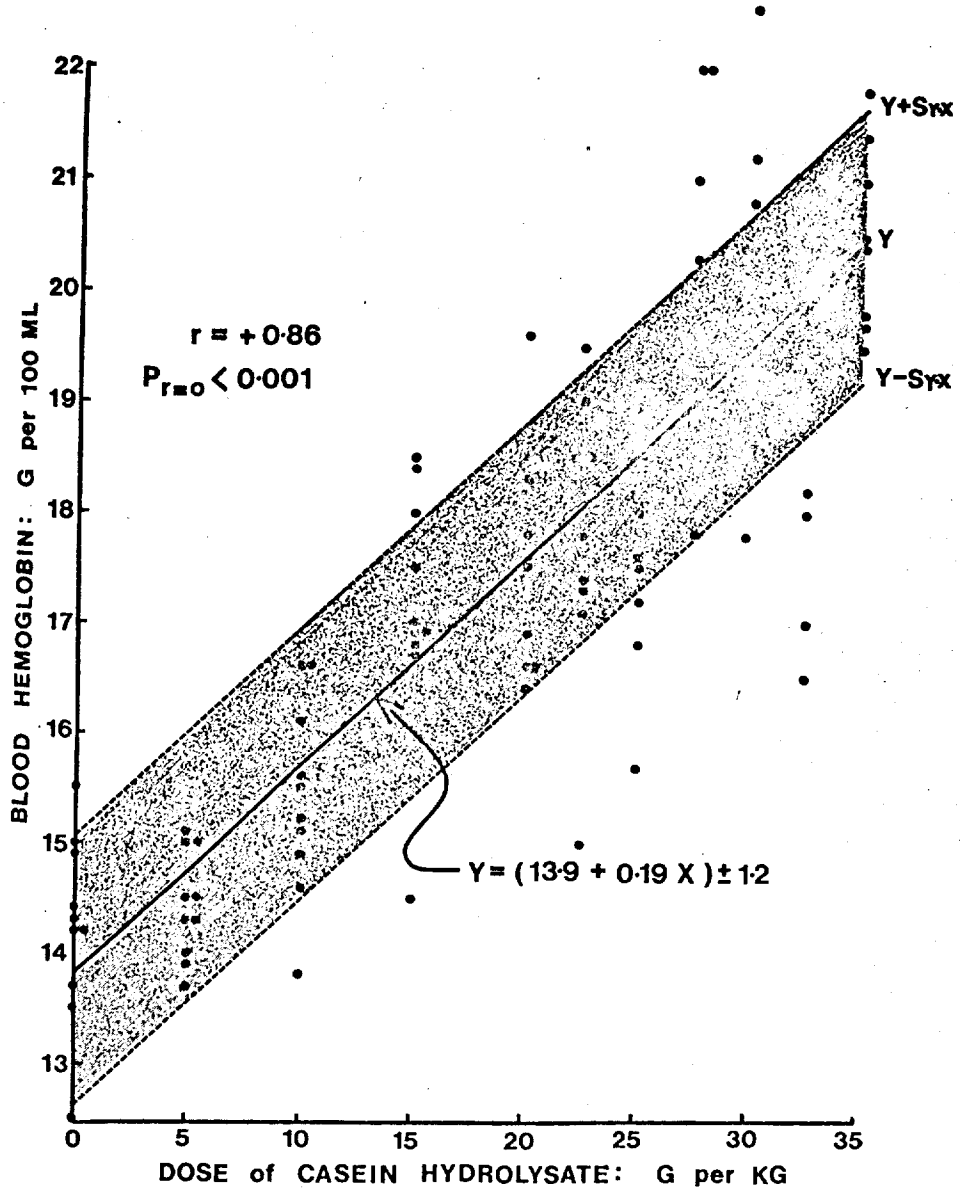

Fig. 2 The regression, on dose of casein enzymatic hydrolysate of values for blood hemoglobin measured at 30 minutes later.

content had reached normal values at 2 weeks and even at one month as indicated by data summarized in tables 1 and 2. All clinical parameters were normal at 2 weeks and one month.

DISCUSSION

The results from casein administration confirm conclusions reached by Bischoff (11) in his review of 1932 and by Hegsted (12) in 1964 that man and animals can

TABLE 1

Changes in the fresh weight of body organs at autopsy in albino rats given doses of casein enzymatic hydrolysate in the range of the oral LD_{50}[1]

Organ	At death (N = 17 + 19 controls)	2-week survivors (N = 15 + 14 controls)	1-month survivors (N = 18 + 16 controls)
Adrenal glands	− 4.3	− 3.8	−12.5 **
Brain	− 3.2 *	− 0.3	+ 0.1
Gastrointestinal tract:			
Cardiac stomach	−24.1 **	−17.7 *	+ 2.0
Pyloric stomach	−21.2 **	− 8.8	− 0.7
Small bowel	− 5.2	− 9.2	+ 9.5 **
Cecum	−19.7 **	+ 4.9	− 0.1
Colon	−18.9 **	−10.8 *	+ 3.6
Heart	− 2.4	+ 2.9	− 3.4
Kidneys	− 5.2 *	− 5.9 *	+ 4.0
Liver	− 6.6	− 5.0	+ 8.4 *
Lungs	+13.6	− 7.6 *	+ 1.5
Muscle (ventral abd. wall)	−30.8 **	− 3.6	−17.5 **
Salivary glands (submax.)	− 2.3	+ 1.5	− 0.6
Skin	−11.2 **	− 0.5	− 2.9
Spleen	−28.4 **	− 8.5	− 7.8
Testes	−17.1 **	− 2.3	− 3.0
Thymus gland	−17.4 *	−11.4 *	− 7.9
Residual carcass	−13.7 **	− 3.7 *	− 0.2
Total body wt	− 2.4	− 3.2 *	− 0.7

[1] The organs were weighed in grams and the results are expressed as mean percent change from controls, specifically as $((\bar{X}_d - \bar{X}_c) \div \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in the drug (casein) treated rats and $\bar{X}_c$ in the respective controls.

* A mean difference significantly different from zero at P = 0.05 to 0.02.

** A mean difference significantly different from zero at P = 0.01 or less.

TABLE 2

Changes in the water content of body organs at autopsy on albino rats given doses of casein enzymatic hydrolysate in the range of the oral LD_{50}[1]

Organ	At death (N = 17 + 19 controls)	2-week survivors (N = 15 + 14 controls)	1-month survivors (N = 18 + 16 controls)
Adrenal glands	−24.6 **	+8.3 *	+7.6
Brain	−15.2 **	−0.3	+0.2
Gastrointestinal tract:			
Cardiac stomach	−41.0 **	−5.3	−0.2
Pyloric stomach	−41.9 **	−2.6	+3.2
Small bowel	−30.3 **	−0.6	+0.8
Cecum	−24.6 **	+0.9	+2.5
Colon	−33.2 **	+1.0	+3.9 *
Heart	−17.3 **	−0.6	−0.2
Kidneys	−17.8 **	−0.7	−0.4
Liver	−17.0 **	+0.5	−6.0 *
Lungs	−16.0 **	−2.2	+1.2
Muscle (ventral abd. wall)	−24.0 **	+0.9	−1.0
Salivary glands (submax.)	−16.2 **	+0.6	+2.5
Skin	−23.6 **	−2.2	−2.7
Spleen	−11.3 **	−0.7	+0.7
Testes	−17.6 **	+1.6 *	+0.6
Thymus gland	−19.5 **	−2.2	+2.3
Residual carcass	−21.3 **	−4.8 *	+3.0

[1] Water content was measured as grams water/100 g dry weight of tissue and the results are expressed as mean percent change from controls, specifically as $((\bar{X}_d - \bar{X}_c) \div \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in the drug (casein) treated rats and $\bar{X}_c$ in the respective controls.

* A mean difference significantly different from zero at P = 0.05 to 0.02.

** A mean difference significantly different from zero at P = 0.01.

TABLE 3

Histopathologic observations in albino rats at death due to oral administration of a lethal dose of casein enzymatic hydrolysate

Organ	Histopathology
Adrenal glands	Sinusoidal erythrocytes packed and distorted; clotting; minute areas of early necrosis
Brain	Marked capillary-venous congestion and hemorrhages in the meninges and brain
Gastrointestinal tract:	
Cardiac stomach	Capillary-venous congestion of the submucosa with areas of lysis of the stratified squamous epithelium
Pyloric stomach	Capillary-venous congestion of the lamina propria and submucosa
Small bowel	Capillary-venous congestion of the lamina propria and submucosa and shrunken villi
Cecum	Capillary-venous congestion and hemorrhage of the lamina propria and submucosa and lysis of glands
Colon	Capillary-venous congestion of the lamina propria and submucosa
Heart	Coronary capillaries and veins congested and occasionally blood clots present
Kidneys	Vascular congestion especially in the loop region and tubular fatty degeneration in late deaths
Liver	Sinusoids packed with distorted erythrocytes and areas of venous clotting
Lungs	Venous clots in early deaths and areas of edema and hemorrhage in late deaths
Muscle	Fibers shrunken but otherwise normal in appearance
Salivary glands (submax.)	Normal appearance
Skin	Ischemic
Spleen	Red pulp shrunken, packed erythrocytes, venous clots
Testes	Tubules shrunken, extravascular clots, some tubular lysis
Thymus gland	Venous clots and some loss of thymocytes

tolerate large amounts of casein in their diets. Hegsted (12) notes that a high protein diet increases the need for water. When young rats in this laboratory (13) were fed a diet of 80% casein for 14 days, there occurred a marked diuresis, some generalized body organ dehydration and an increase in the weight of kidneys and liver but the appearance and growth of the animals were normal.

In the present study, an attempt was made to find a lethal dose of casein given by intragastric cannula to albino rats. Aqueous suspensions coagulated unless they were relatively dilute. The dilute suspensions coagulated and remained in the stomach and repeated administration produced gastric rupture or regurgitation asphyxia in 62 out of 70 animals. Of the 8 survivors, three died apparently from casein intoxication and calculations suggested that the "LD_{50}" of casein would be greater than 1000 g/kg administered over 15 days. Administration of such amounts required the simultaneous administration of distilled water in amounts up to 450 ml/kg per day. The separate administration of this daily volume of water produced clinical signs of toxicity similar to those seen in rats given casein except that there was no aciduria nor any deaths.[14] The widespread vascular congestion of body organs seen in rats dying apparently of casein intoxication was not seen in rats which died

[14] These results were obtained by S. J. Liu of this laboratory in current studies on the effects of daily administration of large volumes of distilled water to albino rats.

TABLE 4

Clinical measurements on survivors of death due to oral administration of casein enzymatic hydrolysate [1]

Measurement	Days after casein		
	1	2	3
	% change		
Body wt, g	− 3.3 ** DD	− 4.1 ** DD	− 1.8 *
Food intake, g/kg/24 hr	− 44.5 **	− 4.9	+ 0.2
Water intake, ml/kg/24 hr	+ 80.8 ** DD	+ 21.2 **	+ 3.6 *
Colonic temperature, °C	+ 0.7 ** DD	+ 0.4	+ 0.1
Urinary volume, ml/kg/24 hr	+ 82.3 ** DD	+ 35.8	+16.8
Urinary pH, 24-hr sample	− 4.4 **	− 0.2	−10.1
Urinary glucose output, mg/kg/24 hr	−100.0 **	+109.0	+36.1
Urinary protein output, mg/kg/24 hr	+494.0 ** DD [2]	− 6.0	−53.2 *
Listlessness, cyanosis, diarrhea, clinical units	0.0	0.0	0.0

[1] The results are expressed as mean percentage change from controls specifically as $((\bar{X}_d - \bar{X}_c) \div \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in the drug (casein) treated survivor and X_c in the controls. Dose-dependence of the mean percentage change is indicatd by "DD."

[2] Urine during the first 24 hours was contaminated with diarrheal casein hydrolysate which may have contributed to the markedly increased output of urinary protein.

* $\bar{X}_d - \bar{X}_c$ significantly different from zero at $P = 0.05$ to 0.02.

** $\bar{X}_d - \bar{X}_c$ significantly different from zero at $P = 0.01$ or less.

of acute water intoxication (14). Death in rats given casein was due to a combination of the toxic effects of water and casein and the LD_{50} of casein is undoubtedly much higher than 1000 g/kg administered over 2 weeks.

The LD_{50} of the soluble sodium and calcium salts of casein was estimated to be some 400 to 500 g/kg given orally to rats over 5 days. The signs of intoxication included some which have been reported in water (14) and in sodium chloride (15) intoxications, mainly in the latter. If large amounts of this preparation were fed in substitution for casein it could produce toxic effects due only in part (if at all) to its casein component.

When casein is hydrolyzed into its component amino acids and polypeptides, it becomes water-soluble and osmotically active. When doses of the order of 25 g/kg were given orally to albino rats in concentrated aqueous solution, they produced a violent gastroenteritis, a withdrawal of water from tissues and blood, widespread capillary-venous congestion, coma and death within a few hours. The syndrome is similar to that found in acute poisoning from sucrose (16) and from concentrated solutions of raw egg white (9). It is obvious that when this preparation is substituted for casein, it should be given in amounts well below the toxic level.

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STUDIES ON EXPERIMENTAL AMYLOIDOSIS

I. Analysis of Histology and Staining Reactions of Casein-Induced Amyloidosis in the Rabbit*

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In the past 35 years amyloidosis has been produced in a number of experimental animals by a variety of techniques. Cattle,[1] horses,[2] rabbits,[3] hamsters,[4] guinea pigs,[5] and mice[6] all have been reported as capable of developing the disease, given the proper stimulus. The latter has consisted of either injection, usually serially, of a variety of substances, or dietary variations, such as cheese supplements or vitamin C deficiency.[5,7] Amyloidosis has also been reported as occurring spontaneously in dogs[1] and in otherwise normal but aged mice of certain strains.[8]

In our laboratory, the induction of amyloidosis in rabbits by means of serial injections of sodium caseinate has provided a reliable and reproducible form of the disorder for study. To date, amyloidosis has been successfully induced in 120 rabbits. Serial bleedings can be carried out with ease and splenic biopsy specimens procured readily. The disease develops eventually to some degree in nearly 100 per cent of injected animals and is histologically similar to the human disorder. The chemical and serologic alterations in the blood of these animals are the subjects of other reports.[9,10]

The present investigation is an analysis of the histologic alterations that occur in different parenchymal organs with the evolution of the disorder. Since a variety of staining techniques have been used in different laboratories in the study of human and experimental amyloidosis, the usefulness of several of these has been compared at the onset and in the final stages of amyloidosis. In addition, fluorescence microscopy and polarization studies have been performed in an effort to characterize amyloid more definitively.

Methods

Thirty-four New Zealand white female rabbits were given subcutaneous injections of 5 ml. of a 10 per cent casein suspension twice

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weekly, utilizing sterile precautions.* The suspensions were freshly prepared each week from commercially available sodium caseinate (obtained from the Matheson Company, Inc.) suspended in distilled water. No antibiotic agents or other preservatives were added. Periodic cultures revealed that while most of the suspensions were sterile, one occasionally exhibited bacterial growth, especially *Bacillus subtilis* and alpha hemolytic streptococcus. The rabbits were maintained on a diet of standard Purina Rabbit Chow (15 per cent protein) and water was administered *ad libitum*. Eight additional rabbits served as controls.

Individual rabbits were sacrificed at intervals of approximately one month. Tissues were fixed in neutral formalin and embedded in paraffin after alcohol dehydration. Serial sections, 5 μ in thickness, were stained with hematoxylin and eosin, Congo red, van Gieson, methyl violet, and crystal violet stains, and by the periodic acid-Schiff reaction. The tissues of 12 rabbits sacrificed after varying intervals were stained with pyronin methyl green. Unstained sections were examined for autofluorescence, using a Reichert ultraviolet microscope at 365 mμ, and for birefringence, using a Spencer polarizing microscope. Congo red stained sections were also examined for fluorescence and birefringence.

The estimation of the amount of amyloid present in a given case was based on the independent appraisals of two observers, using the hematoxylin and eosin, Congo red, crystal violet and van Gieson stained preparations.

Results

General Incidence

Six rabbits were sacrificed after receiving casein injections twice weekly for one month; none showed any signs of amyloidosis (Table I). Eight rabbits were sacrificed at the end of 2 months; 3 of these had amyloid disease of the spleen. Four of the 6 rabbits sacrificed at the end of 3 months of injections also had amyloidosis, as did all rabbits examined after more prolonged periods of casein injections. In 8 control rabbits, sacrificed at bimonthly intervals, there was no evidence of amyloid.

Gross Appearance and Distribution in Various Organs

As seen in Table I and Text-figure 1, the earliest site of deposition of amyloid was in the spleen. Indeed, all spleen sections from rabbits receiving casein for 4 months or more demonstrated amyloidosis. The

* Many of these rabbits served as controls for various other investigations as yet unpublished. This group of animals includes only 7 rabbits mentioned in our previous report concerning serum changes in amyloidosis.[9]

TABLE I

*Incidence and Extent of Casein-Induced Amyloidosis in 42 Rabbits Examined at Monthly Intervals**

Rabbit no.	Months of casein†	Amount of amyloid‡		
		Spleen	Kidney	Liver
164	0	0	0	0
165	0	0	0	0
182	0	0	0	0
184	0	0	0	0
186	0	0	0	0
188	0	0	0	0
191	0	0	0	0
196	0	0	0	0
120	1	0	0	0
127	1	0	0	0
156	1	0	0	0
171	1	0	0	0
175	1	0	0	0
178	1	0	0	0
98	2	2+	0	0
100	2	2+	0	0
111	2	0	0	0
121	2	0	0	0
122	2	0	0	0
136	2	0	0	0
148	2	1+	0	0
150	2	0	0	0
112	3	4+	4+	2+
116	3	3+	1+	0
132	3	3+	0	0
170	3	2+	0	0
172	3	0	0	0
174	3	0	0	0
67	4	4+	0	1+
115	4	4+	2+	2+
97	5	4+	1+	0
99	5	3+	2+	1+
47	6	2+	2+	0
56	6	4+	2+	0
58	6	3+	4+	0
87	6	4+	4+	0
89	6	3+	3+	0
94	6	4+	2+	1+
92	7	4+	2+	1+
59	9	3+	3+	0
14	10	2+	3+	0
8	12	4+	1+	0

* The first 8 rabbits are controls.

† All time intervals adjusted to the nearest month.

‡ Graded as follows:

0 = no amyloid
1+ = 1 to 25 per cent replacement of the organ
2+ = 26 to 50 per cent replacement
3+ = 51 to 75 per cent replacement
4+ = 76 to 100 per cent replacement

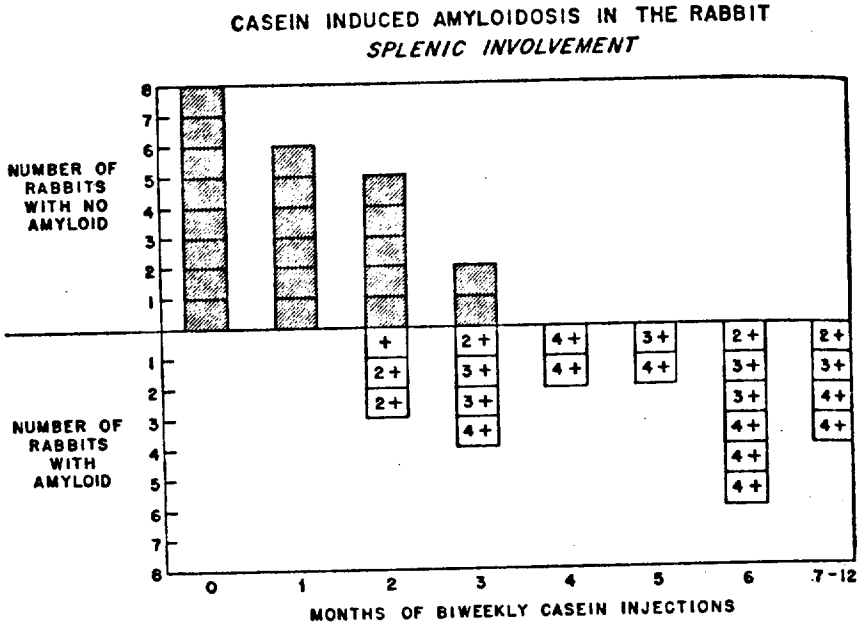
extent of involvement was variable. In several animals receiving the injections for only 3 or 4 months, the spleens showed well over 50 per cent replacement with amyloid. Considerably less was seen in the spleen of one animal which received injections for 10 months.

The normal rabbit spleen was dark red, the weight ranging from 0.5 to 1.5 gm. After one month of injections the spleens were no different in consistency but were slightly larger. The spleens of animals which received casein for longer periods of time and which did develop amyloidosis, were not only larger but firmer and more rubbery. The weight usually was greater with larger amounts of amyloid deposit. For example, one spleen (rabbit No. 100) with 2+ involvement had a weight of 4.2 gm., while that of rabbit No. 99 with 3+ involvement weighed 5.2 gm., and the spleen of rabbit No. 97, with almost total replacement (4+), weighed 6.5 gm.

Renal amyloidosis, primarily glomerular in location, was not found in animals receiving casein for less than 3 months (Text-fig. 2). After 5 months of casein injections, however, renal involvement was invariable. Again, the degree of amyloidosis varied from rabbit to rabbit. One animal, which received casein injections for a full year, exhibited a rather minor degree of renal amyloidosis. Kidneys were weighed in about

one fourth of the cases. No changes in weight were demonstrable with increasing deposition of amyloid.

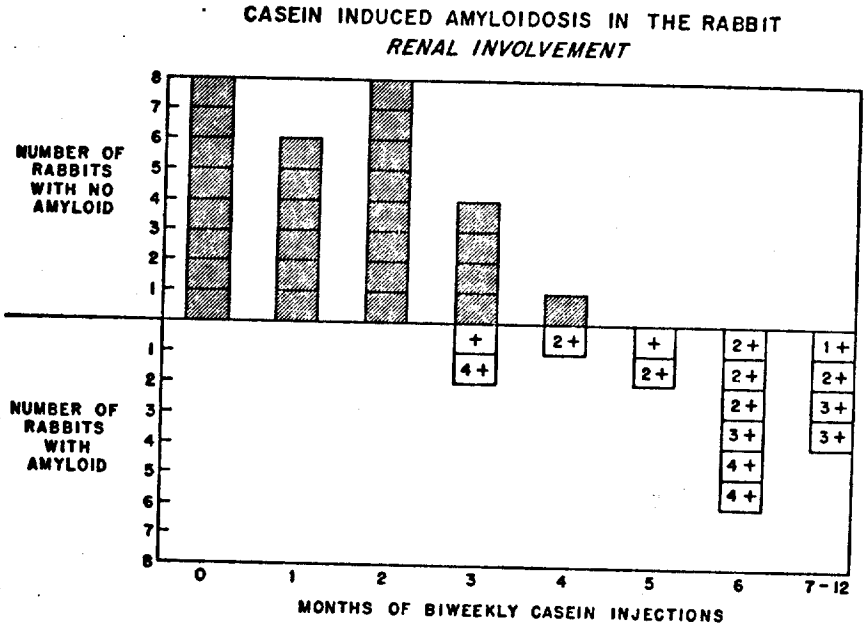
Hepatic amyloidosis (Text-fig. 3) was not marked; the replacement never exceeded 50 per cent of the organ. It was observed only once in rabbits receiving casein for 3 months or less, and was seen in only 5 of 14 animals receiving casein for longer periods. The livers with amyloidosis were also more firm, dark and rubbery. Weights of this organ were not recorded routinely. Lymph node involvement was occasionally encountered, but amyloidosis of the heart, skeletal muscle, gastrointestinal tract, or skin was not observed. Endocrine organs were not regularly examined.



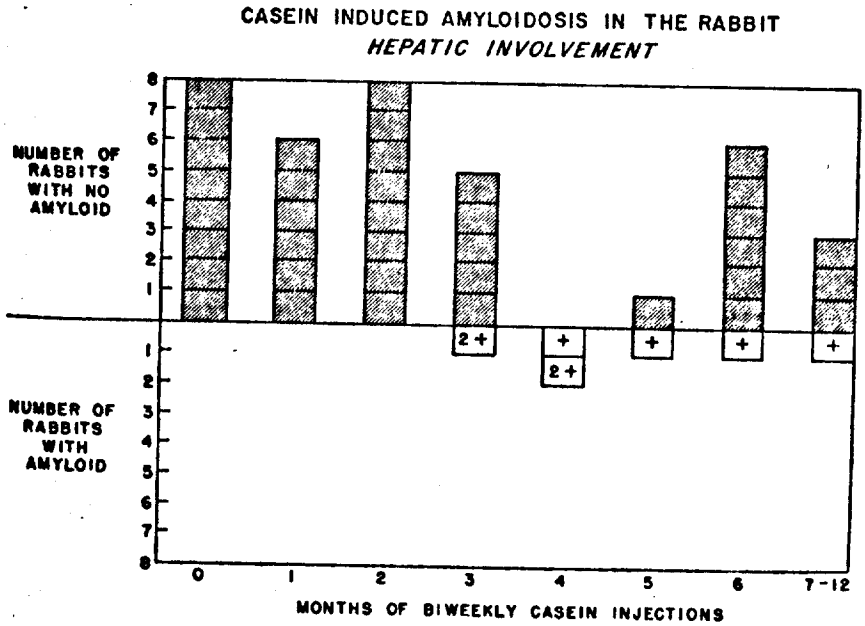
Text-figure 1. The extent of amyloid is graded 0 to 4+, as described in the footnote to Table I.

Histologic Appearance

Spleen. The amyloid initially appeared in the marginal area of the red pulp, i.e., perifollicular region (Figs. 1 and 2). Here one observed a structureless, apparently amorphous, eosinophilic substance which bound Congo red and stained metachromatically with crystal violet. The amyloid accumulated extracellularly in the subendothelial region. An increase in plasma cells and reticuloendothelial cells was frequently seen. Occasionally, the latter contained PAS-positive granules. The heterophils (pseudo-eosinophils in the rabbit) were also increased in



Text-figure 2. The extent of amyloid is graded o to 4+, as described in the footnote to Table I.



Text-figure 3. The extent of amyloid is graded o to 4+, as described in the footnote to Table I.

numbers after one to two months of casein injections. The granules of the latter cells in both treated and control animals bound Congo red, and were distinct from the PAS-positive granules.

Occasionally, in the early stages of the process, isolated nodular deposits in the red pulp or small intrafollicular deposits could be observed. With time and increasing numbers of casein injections, the amyloid appeared to replace most of the red pulp, sparing only trabeculas, portions of the lymphoid follicles, and the capsule (Figs. 3 and 4). In several animals one could observe a small focus of more intensely eosinophilic substance indistinguishable from "fibrinoid" in the red pulp (Fig. 3). This focus bound Congo red weakly, was orthochromatic with crystal violet and stained dark yellow with the van Gieson stain.

In the animals with the most advanced lesions, there was almost total obliteration of the splenic architecture to a point where the sections were no longer recognizable as spleen. The red pulp was replaced by a relatively acellular, amorphous, glassy material. During the development of the amyloidosis, no evidence of simultaneous reabsorption was noted. In only one section was a giant cell observed.

The amyloid substance exhibited similar staining characteristics in all stages of the disease. It was eosinophilic, bound Congo red, and was metachromatic with crystal violet. It stained olive yellow with the van Gieson and light purple with the periodic acid-Schiff stains. Fluorescence microscopy demonstrated a faint blue-green autofluorescence at 365 mμ and a pink fluorescence after staining with Congo red. Polarization microscopy showed a faint birefringence of the unstained, formalin-fixed sections. Staining with Congo red intensified the birefringence.

Kidney. The earliest lesions of amyloidosis in the kidney occurred in the glomerular tufts, where hyaline deposits of eosinophilic substance were found. The distribution of lesions among the glomeruli was fairly even. Further deposition of amyloid within the glomerular tufts resulted in their almost complete replacement (Figs. 5 and 6). The final stage was one of sclerosis and obliteration of glomeruli. Under high magnification, the glomerular deposits appeared in close relationship to the capillary endothelium. Accumulations of amyloid were seen between the endothelial cells and the basement membrane. Occasionally, endothelial cells appeared to be completely surrounded by amyloid (Fig. 6). The capillary lumen in advanced cases was all but obliterated.

Simultaneously with the evolution of the glomerular lesion, similar but less extensive accumulations of amyloid occurred in the interstitial

tissues of the kidney. Most commonly the medullary connective tissue harbored discrete deposits of amyloid, although occasionally subcapsular or nodular deposits could be found in the cortex. The lesion appeared to be most severe in the region of the corticomedullary junction (Fig. 7). Rarely was the interstitial deposit as marked as that in the glomeruli. High-power views of the medullary deposits also showed a close relationship to capillary basement membranes and interstitial connective tissue elements. The staining characteristics of the renal deposits were identical with those in the spleen.

With the PAS stain, the capsular as well as the glomerular basement membranes appeared thickened. Occasionally, within Bowman's space, "crescents" were observed. These were eosinophilic, and orthochromatic when stained with crystal violet; they did not bind Congo red, and appeared yellow with the van Gieson stain. Late in the disease, colloid casts were often present in the tubules. The casts, too, were eosinophilic and did not bind Congo red. With one exception, they were orthochromatic when stained with crystal violet. They usually were stained bright violet with PAS.

Liver. In the series of 34 rabbits treated with casein, only 6 showed hepatic deposit of amyloid, and usually this was not marked. The distribution was periportal in 2 cases, and more diffuse in the remaining 4 (Fig. 8). Two of the latter had a tendency to more marked involvement of the centrilobular region. The amyloid appeared to accumulate between the parenchymal and the Kupffer cells. With progressive deposition, the parenchymal cells were obscured and were replaced by nodular masses of amyloid. The staining characteristics of the amyloid in the liver were identical with those in the spleen and kidney.

Discussion

The observations reported are of interest in two respects. First, they illustrate the histologic sequence in the development of amyloidosis, and second, they provide an opportunity to evaluate the usefulness of various staining and optical methods in the detection of the substance and to learn more of its nature.

Histologic evidence of abnormal activity in the spleen was apparent during the course of casein injections, even before the first appearance of amyloid. Reticuloendothelial proliferation was noted in the marginal zone of the red pulp by the end of one month. At that time increased numbers of plasma cells and heterophils were observed. In some instances PAS-positive, and in rare cases, pyroninophilic substance was found in the reticuloendothelial cells. None of these features, however, were consistently observed in all animals at the end

of one or two months' time, and they varied greatly in magnitude.

The relation of any one of the observations to the pathogenesis of amyloid is uncertain at present. Although plasma cell proliferation is often associated with antibody formation, there are conflicting reports in the literature as to the role immunologic phenomena play in the genesis of amyloid.[11,12] The increase in the number of heterophils noted was far more impressive than the increase in plasma cells. The fact that the granules of the heterophils (pseudo-eosinophils) bound Congo red was considered to be a nonspecific feature since the heterophils of untreated controls exhibited an identical staining reaction.

It was of interest that the greatest and earliest increase of heterophils and plasma cells, and, indeed, the earliest appearance of amyloid, was in the marginal zone of the red pulp. This region has been described as functionally transitional between red and white pulp. It has been said to be a very active part of the spleen, and perhaps the portion controlling blood cell sequestration and other splenic adaptive functions.[13,14]

The presence of PAS-positive and pyroninophilic substances in the spleen has been noted and extensively discussed by Teilum.[15] He regarded them to be indicative of glycoprotein synthesis. This assumption is supported by the fact that the animals, by the end of one month, also demonstrated marked increase in serum hexosamine concentration, indicating increased concentration of serum glycoproteins.[9] On the other hand, in the present investigation, the amount of PAS and pyroninophilic material was far less than that described by Teilum. Some of these differences may be accounted for by differences in histologic technique. However, the evidence that these histologic features *per se* denote abnormal glycoprotein synthesis would seem to be circumstantial. The conclusion that simply because the accumulation of PAS-positive and pyroninophilic material precedes the development of amyloidosis, the former substance is a precursor of the latter does not appear to be justified. The nonspecific nature of the staining reactions and the lack of understanding of their chemical nature make it difficult to appreciate their basic significance.

Histologic evidence of renal amyloidosis was first seen in one rabbit after 3 months of injections, but in most animals it was delayed until the fourth or fifth month. In Richter's[16] studies of amyloidosis induced in rabbits by sodium ribonucleate, the renal lesions also occurred much later than in the spleen. Colloid casts were present in 11 of 15 rabbits with kidney amyloidosis and in 2 of the animals with no renal amyloid. In all instances the casts were eosinophilic, orthochromatic,

strongly PAS positive, and failed to stain with Congo red. These reactions suggest that these proteins differ from amyloid.

Hepatic involvement in this series was minimal. When small accumulations of amyloid did occur, they were localized between Kupffer and parenchymal cells. These observations are similar to those previously reported. The overall distribution in casein-induced amyloidosis of rabbits is, therefore, parenchymal—resembling the so-called "secondary" amyloidosis in human subjects.

The rabbits with experimental amyloidosis provide an excellent opportunity for investigation of the histologic characteristics of this substance in different stages of its development. The appearance of the amyloid was identical from organ to organ and did not vary with duration. The deposit in the spleens of animals sacrificed at 2 to 3 months stained identically with that in those sacrificed at 6 to 8 months. It had similar characteristics in all organs no matter what the time of sacrifice.

Evaluation of Histologic Techniques

Hematoxylin and Eosin. Apart from its obvious use in conventional tissue evaluation, this stain was often sufficiently distinctive to make one suspect amyloid strongly in moderately and severely affected organs. Its use alone, however, was never felt to be conclusive in view of the possibility of confusion of amyloid with other extracellular eosinophilic deposits of different nature. In addition, minimal amounts of amyloid could be overlooked easily.

Congo Red. The use of this dye in the histologic demonstration of amyloid is classic. Nonetheless, it proved to have certain disadvantages in our hands. Unless the sections were carefully decolorized, dense collagenous tissue often bound considerable amounts of the dye. Moreover, the faintness of color precluded its use in fully appraising minimal depositions. As will be described subsequently, however, fluorescence and polarization studies after Congo red staining were more significant.

Metachromatic Dyes. Although metachromatic dyes have been known since 1875, they are only now being characterized.[17] Metachromasia is a subject of controversy because of the complex nature and often impure composition of the dyes involved. However, as a tool in the diagnosis of amyloidosis, it has proved to be of great value. In the present investigation, methyl violet (color index, 680) was first used to test for metachromasia. However, because of occasional variations in staining and the impure composition of this dye

(this has been corroborated by Dr. Børge Larsen, using paper chromatography), crystal violet (color index, 681) was then utilized. It, too, was shown by chromatograms to contain two distinct spots, but despite this, results were technically reproducible. The metachromasia of amyloid was a most useful property in locating early and minimal deposits. In the spleen, rare areas of "fibrinoid" stained orthochromatically. These had a denser, coarser structure which was quite distinguishable from the glassy appearance of amyloid. In addition, these lesions did not bind Congo red.

Van Gieson Stain. In 1889, van Gieson described a dye useful in staining the connective tissue of peripheral nerves.[18] Since then, the value of this dye in differentiating collagen (which stains red) from other connective tissue depositions has become well known. Although in the present investigation the van Gieson stain did not serve to establish the diagnosis of amyloid in any animal, it was useful in differentiating amyloid from collagen. Amyloid in the rabbit stained yellow with this dye and had less of the khaki or orange tint that has been observed in human amyloidosis. The staining quality was also consistent with the observation that amyloid does not contain a significant amount of hydroxyproline, and thus supports the inference that amyloid is not primarily collagenous in nature.[19]

Periodic Acid-Schiff (PAS) Reaction. Although amyloid stained in weakly positive manner with PAS, the use of this dye did not aid in the detection of this substance.[20] During the development of the amyloid, PAS-positive granules were occasionally observed in the reticuloendothelial cells of the spleen. These were not necessarily related to the evolution of the disease.

Pyronin Methyl Green Stain. The tissues of 2 normal and 12 casein-treated rabbits containing varying degrees of amyloidosis were stained with pyronin methyl green.[15] Occasional pyroninophilic cells were observed, but the phenomenon was not striking. It bore no clearcut chronologic or qualitative relationship to the degree of amyloidosis.

Fluorescence Studies. Unstained, formalin-fixed sections, 5 μ in thickness, demonstrated that amyloid substance was autofluorescent when viewed at a wave length of 365 mμ. This did not, however, enable it to be clearly distinguished from other tissue components. Following Congo red staining, however, amyloid exhibited a pink fluorescence. This was distinctive and sharply localized to the amyloid in the well decolorized specimen. When sections were thick, showed folds, or the dye was not adequately decolorized, false positive dye fixation was frequently observed. However, the technique of Congo red staining

and fluorescence microscopy, when carried out meticulously, was useful in delineating small accumulations of amyloid.

Polarization Microscopy. Examination of unstained, formalin-fixed sections demonstrated that amyloid was very weakly birefringent with respect to the long axis of the deposit under observation. As observed by Missmahl and Hartwig,[21,22] the birefringence increased markedly after Congo red staining. In contrast to collagen, which is white on visualization in the polarizing microscope, the unstained and Congo red stained amyloid had a pale green hue. As in the case of collagen, however, the birefringence was positive with respect to the long axis of the deposit. This anisotropy suggests that amyloid may have an orderly intrinsic molecular arrangement accentuated by Congo red binding, and is not completely amorphous in nature.

Missmahl believed that amyloid contained collagen fibers which accounted for its behavior in polarized light. On the other hand, it is possible that its behavior is due to an intrinsic orientation of micelles that compose the amyloid itself. The chemical analyses previously mentioned,[19] however, would seem to indicate that collagen was not a significant part of amyloid. Since birefringence in the present investigation was positive with respect to the long axis of the deposit, it would appear that the submicroscopic units that make it up are oriented parallel to its long axis. More detailed studies of the fine structure of amyloid by electron microscopic methods have been carried out and are the subject of other reports.[23-25]

Summary and Conclusions

1. Amyloidosis was induced in 34 rabbits by subcutaneous casein injections twice weekly for periods up to 12 months. Animals were sacrificed at monthly intervals in order to investigate the sequence of alterations and the magnitude of organ involvement. Eight additional rabbits served as controls.

2. All animals which received the injections for 4 or more months exhibited amyloidosis of some degree.

3. The spleen was affected in most animals after 2 months and was invariably laden with amyloid after 3 to 4 months. The kidney was involved progressively only after 4 to 5 months of injections. The liver was uncommonly affected and contained smaller deposits of amyloid.

4. Tissues were stained with hematoxylin and eosin, Congo red, PAS, van Gieson, crystal violet, and pyronin methyl green stains. In addition, unstained and Congo red stained sections were examined for fluorescence and birefringence.

5. The combination of hematoxylin and eosin, crystal violet and van Gieson stain was most useful in detecting amyloid. Examination with ultraviolet light after Congo red staining was useful in detecting minimal deposits.

6. Polarization studies demonstrated positive birefringence in amyloid, suggesting that it may have an organized molecular rather than an amorphous structure.

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[*Illustrations follow*]

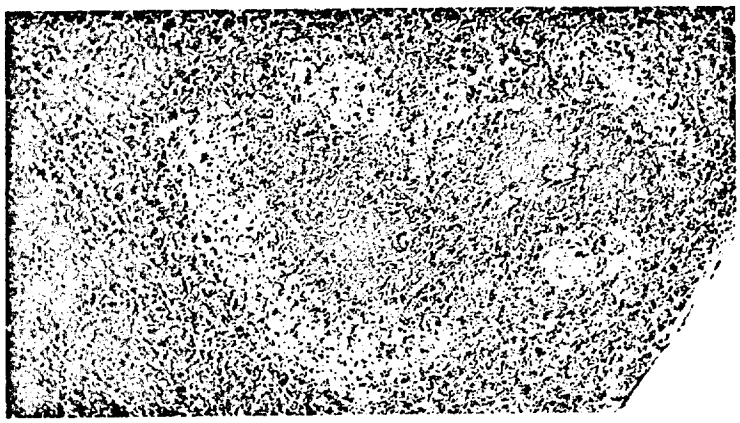
LEGENDS FOR FIGURES

All sections illustrated were stained with hematoxylin and eosin.

FIG. 1. Spleen of rabbit No. 148 (Table I). Perifollicular distribution of amyloid; an early stage of involvement, classified as 1+. × 80.

FIG. 2. Higher magnification of Figure 1, demonstrating amyloid in the marginal zone of the red pulp. Amyloid appears to be located in the subendothelial region. × 450.

FIG. 3. Spleen of rabbit No. 99 (Table I). Lymphoid follicle, perifollicular amyloid (3+) and darker staining "fibrinoid" (F) amidst the amyloid. × 125.



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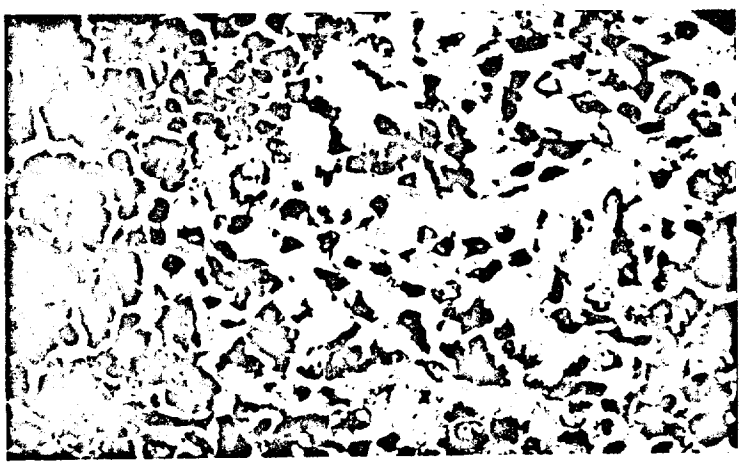

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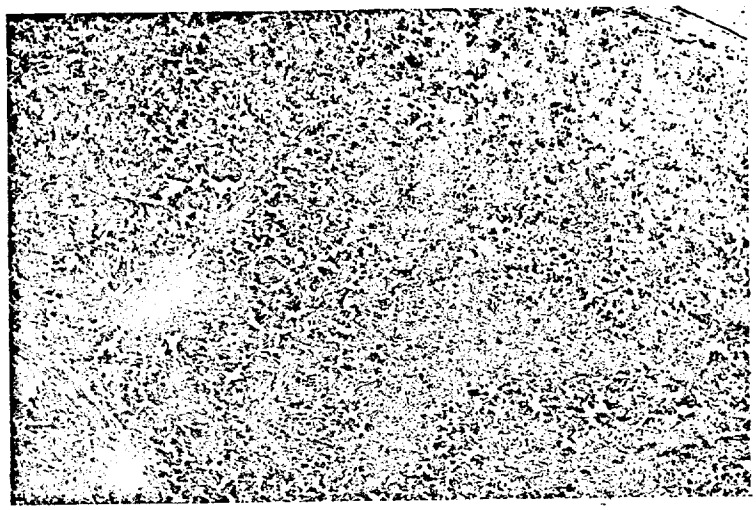
FIG. 4. Spleen of rabbit No. 50, with advanced amyloidosis (4+). Almost total replacement of the organ by amyloid with only scattered remnants of trabeculas and lymphoid follicles. × 37.

FIG. 5. Kidney of rabbit No. 58. Advanced amyloidosis of renal cortex, showing almost complete replacement of glomeruli with amyloid. Tubular casts are present in the medullary rays. × 80.

FIG. 6. Kidney of same rabbit. Glomerulus with advanced amyloidosis. Amyloid appears to be localized between the basement membrane and endothelial cells of the glomerulus, with the latter occasionally surrounded by amyloid substance. × 450.



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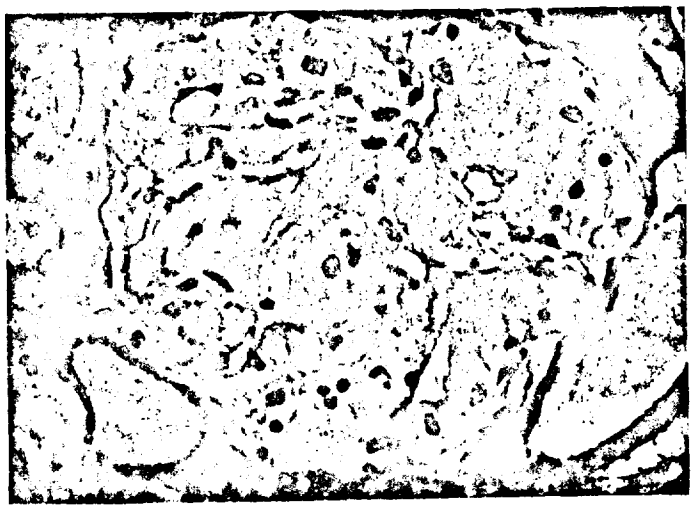

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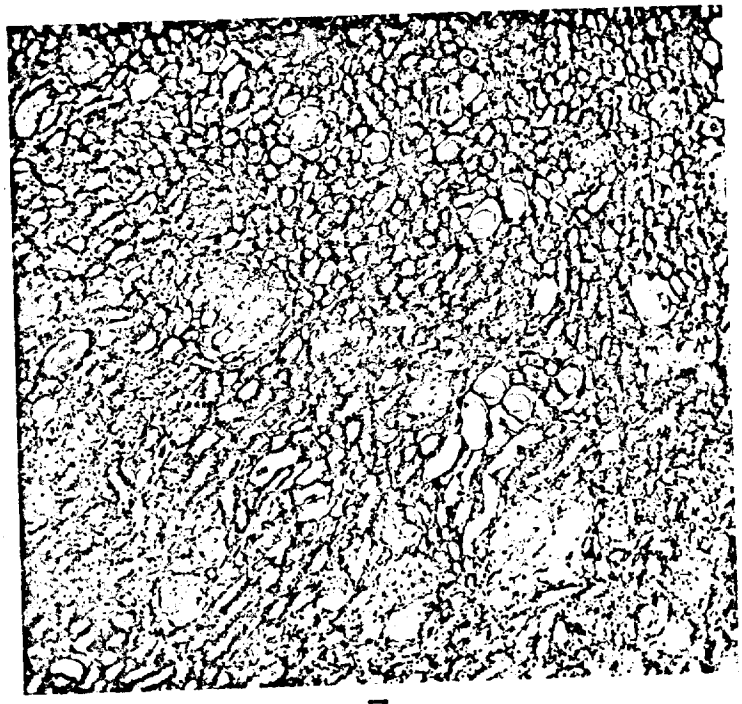
FIG. 7. Same rabbit kidney, showing amyloidosis of renal medulla. Involvement is most severe at the corticomedullary junction. Many tubular casts are also present. × 80.

FIG. 8. Liver of rabbit No. 115. Small amounts of amyloid in periportal and centrilobular regions. Parenchymal cell destruction may be seen in areas of amyloid deposition. ×



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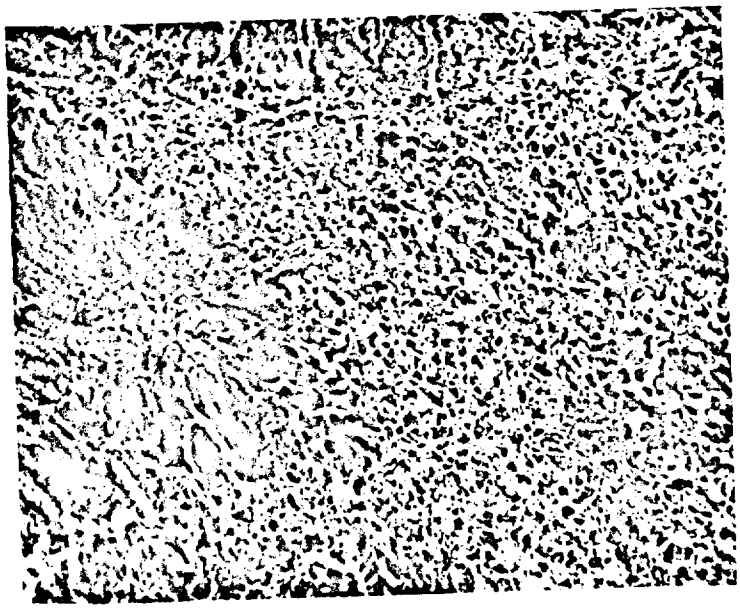

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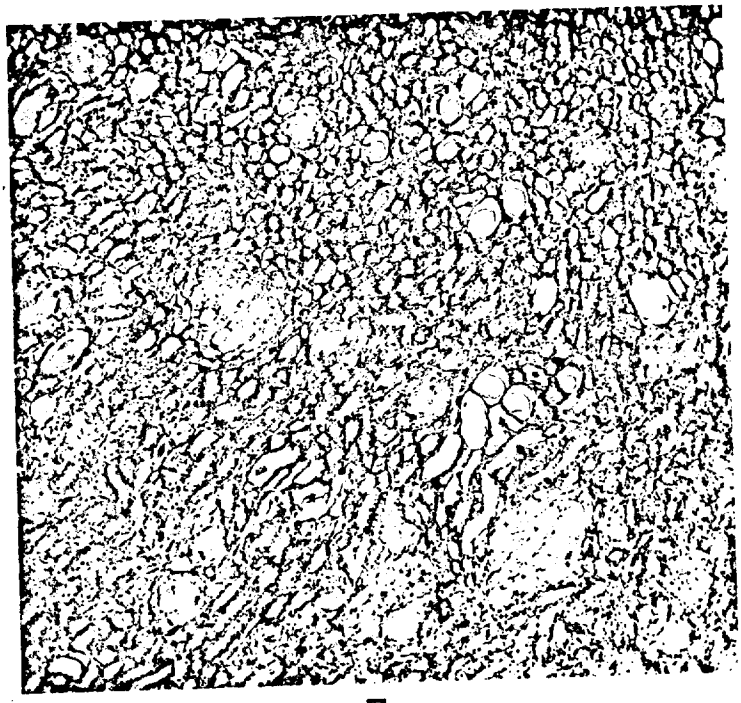

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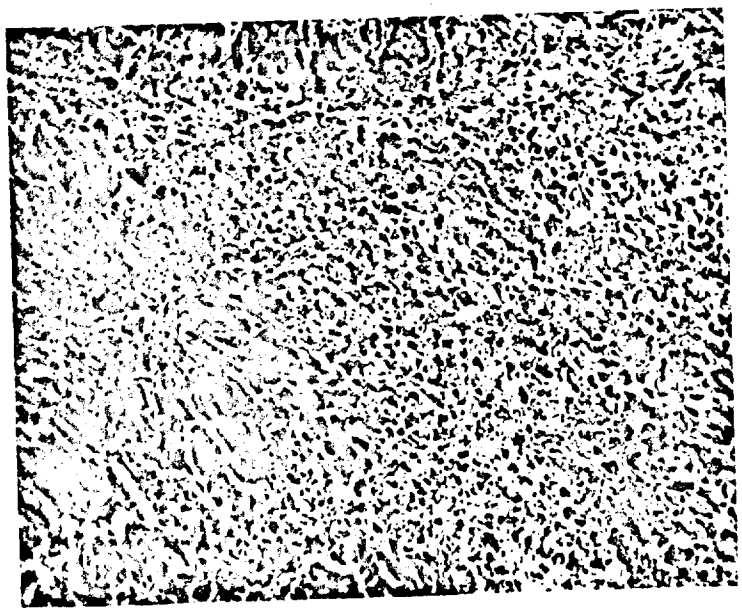

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J. Nutr. 104: 151-159, 1974

Effect of Abomasal Infusion of Sodium Caseinate on Milk Yield, Nitrogen Utilization and Amino Acid Nutrition of the Dairy Cow[1]

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ABSTRACT Previous studies have not established the sequence of limiting amino acids for the lactating cow or the mode of response from postruminal casein administration. Therefore, an evaluation of metabolite uptake by the lactating bovine mammary gland has been used to suggest a possible sequence of limiting amino acids for milk protein synthesis. Seven lactating Holstein cows were used to study the effect of abomasal versus ruminal infusion of sodium caseinate on milk production, milk composition, nitrogen utilization, levels of various blood metabolites and metabolite uptake by the mammary gland. Milk yield, milk nitrogen and nitrogen retained were significantly increased and fecal and urinary nitrogen were significantly decreased when sodium caseinate was infused postruminally. A general increase in the jugular blood concentration of all essential amino acids and most of the nonessential amino acids was observed when sodium caseinate was infused into the abomasum. The estimated uptake of each amino acid from the blood and the theoretical quantity of milk protein which could be synthesized by the mammary gland suggest phenylalanine, methionine, lysine, threonine, etc., to be the sequence of limiting essential amino acids for the lactating dairy cow. J. Nutr. *104:* 151–159, 1974.

INDEXING KEY WORDS amino acids · nitrogen metabolism · dairy cattle nutrition

Studies are being conducted to determine nutrients which may be limiting milk production in high producing dairy cows and to elucidate practical methods of supplying these nutrients in the diet for optimum performance. Glucose, protein and amino acids have been suggested as the nutrients most likely to be limiting milk production and milk protein synthesis (1, 2). Increased wool growth and nitrogen retention have been observed in sheep when casein or amino acids were infused into the abomasum (3).[2] Milk production and/or milk constituents were increased when glucose and proteins were administered into the abomasum (4)[3-5] or when methionine hydroxy analog was fed in the diet (5, 6) of lactating cows. Nitrogen retention was increased in steers when casein or a mixture of amino acids were infused into the abomasum.[2]

We have continued efforts to establish the limiting amino acid(s) for milk protein synthesis and milk production in the lactating dairy cow. The research approach used in most other laboratories has been to infuse proteins or amino acids into the abomasum and to use jugular plasma concentrations of amino acids for suggesting limiting amino acids. Determination of lim-

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[1] Supported in part by the Illinois Agricultural Experiment Station.
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iting amino acids for lactating cows by measuring jugular plasma concentrations of amino acids has not been successful. In contrast to this approach, we have conducted experiments to determine the effect of infusing casein into the abomasum on milk production, milk composition and nitrogen utilization in the lactating cow and compared these responses to arterial plasma metabolite levels and to metabolite uptake by the mammary gland.

EXPERIMENTAL PROCEDURE

The trial was divided into three 7-day experimental periods during which sodium caseinate was infused into the rumen, abomasum and rumen. Approximately 5 liters of a 9% w/v solution of sodium caseinate was continuously infused during each 24-hour period. Seven rumen-fistulated mid-lactation Holstein cows, producing 15.1 to 37.9 kg of milk per day, were used as experimental animals. The diet consisted of alfalfa hay (19.7% crude protein) and a 14% crude protein concentrate mixture. The concentrate mixture consisted of 84.25% ground shelled corn, 13.00% soybean meal, 1.50% dicalcium phosphate, 1.20% trace mineral salt,[6] and 0.05% vitamin supplement.[7] Approximately 1 kg of concentrate was fed at the time of milking for each 3 kg of milk produced and hay was fed ad libitum. Sodium caseinate was infused into the abomasum through a 6-mm polyethylene tube attached to a 60-ml bottle. The tube was passed through the rumen cannula and sulcus omasi into the abomasum. Feed refusals were measured daily. Concentrates, hay and feed refusals were sampled daily and analyzed for dry matter content in a forced air oven at 55°. Total collection of feces and urine for determination of digestibility and nitrogen balance were made during the last 5 days of each infusion period. Urine was collected by means of indwelling catheters inserted into the bladder (7). Urine, collected under 6 N HCl, was measured and sampled daily. Feces were collected, sampled and dried daily at 55°. Cows were milked twice daily and a daily composite milk sample was taken for crude protein (N × 6.38), fat and lactose determinations. Crude protein determinations of feed, feces, urine and milk were by the Kjeldahl method, milk fat by the Babcock method, and lactose according to the procedure of Coffey and Reithel (8).

On the last day of each of the three infusion periods (approximately 4 hours postfeeding and 5 hours postmilking) blood samples were collected into heparinized blood tubes from the jugular vein, the internal iliac artery (9) and the caudal superficial epigastric vein. Hematocrits were determined by centrifugation in a microhematocrit centrifuge tube for 5 minutes. Plasma was deproteinized by adding 10 ml of plasma to 1 ml of a solution containing 500 mg of sulfosalicylic acid and 2.5 μmoles of norleucine as an internal standard. The supernatant obtained by centrifugation was combined with polyethylene glycol (M.W. 400) in a 5:1 ratio and stored at −195.8° (liquid nitrogen) until analyzed for plasma free amino acids on an automatic amino acid analyzer.[8] Research in our laboratory indicated that storage losses of methionine were eliminated upon storage at this temperature.[9] Plasma urea was determined according to Chaney and Marback (10) and plasma glucose was assayed by the glucose oxidase method.[10]

Blood flow through the mammary gland was estimated using the regression equation reported by Kronfeld et al. (11). Uptake of amino acids, urea and glucose from blood transversing the mammary gland was calculated by multiplying estimated blood flow per 24 hours by the difference in plasma arterial and venous concentrations. The percentage arterial concentration of each amino acid extracted by the lactating mammary gland was calculated by dividing the mammary arteriovenous difference by the arterial concentration and multiplying by 100.

Amino acid composition of milk protein (12) was used to calculate the theoretical quantity of milk protein that could be

[6] Contains: (minimum amount) iodine, 0.005%; iron, 0.125%; copper, 0.025%; cobalt, 0.005%; manganese, 0.200%; zinc, 0.250%; and salt, 98.5% (maximum) or 96.0% (minimum). Purchased from Gunther Salt Co., St. Louis, Mo. 63102.

[7] M & R Premix no. 177. Contains: 10,000 USP units retinylacetate and 1,250 USP units ergocalciferol per gram. Purchased from Vitamins Inc., Chicago, Ill.

[8] Phoenix Amino Acid Analyzer, model K-8000. Virtis Co., Gardiner, N. Y. 12525.

[9] Clark, J. H. & Derrig, R. G. (1971) Unpublished data.

[10] Glucostat kit, Worthington Biochemical Corp., Freehold, N. J. 07728.

TABLE 1

Dry matter intake, milk production, and milk composition

Variable	Site of sodium caseinate infusion		
	Rumen	Abomasum	Rumen
D.M. intake, kg/day			
Hay	6.6 ±0.66[1]	6.9 ±0.66	6.8 ±0.66
Concentrate	7.9 ±0.69	7.9 ±0.69	7.9 ±0.69
Sodium caseinate	0.4 ±0.01	0.4 ±0.03	0.4 ±0.03
Total	14.9 ±1.11	15.2 ±1.28	15.1 ±1.25
D.M. digestibility,%[2]	67.3 ±1.2	68.6 ±1.3	65.3 ±1.1
Milk, kg/day[3]	23.4 ±2.7	24.6 ±2.8	23.2 ±2.9
4% fat-corrected milk, kg/day	20.8 ±2.0	21.5 ±2.2	20.9 ±2.3
Milk fat,%	3.35±0.19	3.15±0.10	3.39±0.15
Milk protein, %[3]	3.05±0.11	3.24±0.11	3.12±0.08
Milk lactose,%	5.18±0.19	5.19±0.07	5.37±0.36

[1] All values represent mean of seven cows ±SEM except for milk lactose which represents mean of four cows ±SEM. [2] Increased by abomasal infusion, $P < 0.05$. [3] Increased by abomasal infusion, $P < 0.01$.

synthesized. Theoretical milk protein was calculated by multiplying each amino acid uptake for a 24-hour period by the number of grams of milk protein synthesized per gram of each amino acid. This value represents the calculated quantity of milk protein that could be synthesized if the total quantity of the amino acid extracted was incorporated into milk protein. Kjeldahl nitrogen content of the milk produced and amino acid composition of milk (12) were used to estimate the amino acid output in milk.

Data were analyzed statistically by a comparison of treatment means (rumen versus abomasum infusion) (13).

RESULTS AND DISCUSSION

No significant differences were detected for dry matter intake, milk fat percentage, 4% fat corrected milk production, or percentage lactose in the milk when sodium caseinate was infused into the abomasum or rumen (table 1). Dry matter digestibility, average daily milk yield, milk protein percentage (table 1) and grams of milk nitrogen (table 2) were significantly increased when sodium caseinate was infused per abomasum.

Similar studies by Vik-Mo et al.[4, 5] showed an increase in milk production and milk protein percentage when casein was infused per abomasum in lactating dairy cows. Broderick et al. (4) infused 800 g of casein into the abomasum and observed an increase in milk protein and a nonsignificant increase in milk yield but a decrease in feed intake. In contrast to our findings, the decreased feed intake observed by Broderick et al. (4) may be attributed to the large amount of casein infused. Our results as well as those of Vik-Mo et al.[4, 5] and Broderick et al. (4) suggest that supplying a mixture of amino acids via casein

TABLE 2

Average daily nitrogen intake and utilization

Variable	Site of sodium caseinate infusion		
	Rumen	Abomasum	Rumen
N intake, g			
Oral	399±34[1]	409±38	403±37
Infused	57± 2	59± 1	59± 1
Total	456±34	468±39	462±38
N absorbed, g[2]	319±23	339±28	313±25
N excreted, g			
Feces[3]	137±14	129±12	149±13
Urine[3]	193± 8	181±14	202±15
Milk[4]	109± 9	123±10	112±11
N retained, g[4]	17± 9	35± 8	−1± 5
Productive N, g[4,5]	126±16	158±17	111±12
N, % intake			
Feces[6]	30± 1	28± 1	32± 1
Urine[6]	42± 2	39± 2	44± 1
Milk[4]	24± 1	26± 3	24± 1
Retained[4]	4± 2	7± 1	−1± 1
Productive[4]	28± 2	33± 2	24± 1
N, % absorbed			
Urine[6]	61± 2	54± 2	65± 2
Milk	34± 1	36± 1	36± 1
Retained[2]	5± 3	10± 2	−1± 2
Productive[4]	39± 2	46± 2	35± 2

[1] Values represent mean of seven cows ±SEM. [2] Increased by abomasal infusion, $P < 0.05$. [3] Increased by ruminal infusion, $P < 0.05$. [4] Increased by abomasal infusion, $P < 0.01$. [5] Milk nitrogen plus nitrogen retained. [6] Increased by ruminal infusion, $P < 0.01$.

TABLE 3

Jugular plasma amino acid, glucose, and urea concentrations

Variable	Site of sodium caseinate infusion		
	Rumen	Abomasum	Rumen
		mg/100 ml	
Lysine[2]	0.95±0.09 (10)[1]	1.31±0.12 (9)	0.88±0.09 (9)
Histidine[2]	0.78±0.08 (8)	1.01±0.06 (7)	0.71±0.09 (8)
Arginine[3]	1.21±0.12 (13)	1.38±0.17 (10)	1.18±0.13 (13)
Isoleucine[2]	1.02±0.06 (11)	1.85±0.33 (13)	1.05±0.11 (11)
Leucine[2,4]	1.48±0.13 (16)	2.59±0.48 (19)	1.42±0.19 (16)
Threonine[2,3]	1.03±0.11 (11)	1.14±0.18 (8)	1.00±0.07 (11)
Valine[4]	1.97±0.14 (21)	3.37±0.25 (25)	2.09±0.19 (23)
Methionine	0.29±0.01 (3)	0.43±0.09 (3)	0.31±0.03 (3)
Cystine	0.41±0.06	0.34±0.08	0.38±0.06
Phenylalanine[3,5]	0.59±0.05 (6)	0.73±0.05 (5)	0.61±0.06 (7)
Tyrosine[2]	0.68±0.06	0.88±0.05	0.69±0.05
Alanine[5]	1.77±0.05	1.96±0.08	1.75±0.12
Aspartic acid	0.12±0.03	0.19±0.06	0.09±0.01
Glutamic acid	0.76±0.13	0.76±0.06	0.56±0.05
Glycine	2.31±0.23	2.30±0.26	2.01±0.21
Serine[5]	0.82±0.05	0.94±0.06	0.74±0.04
Essentials as % of total[6]	57.5	65.3	59.8
Glucose	77.5 ±2.2	76.3 ±1.8	70.8 ±1.8
Urea	20.2 ±1.9	18.4 ±1.8	18.8 ±1.2

[1] Values are mean of seven cows ±SEM. Numbers in parentheses are % of total essential amino acids. [2] Increased by abomasal infusion, mg per 100 ml ($P < 0.01$). [3] Increased by ruminal infusion, % of essentials ($P < 0.01$). [4] Increased by abomasal infusion, % of essentials ($P < 0.05$). [5] Increased by abomasal infusion, mg per 100 ml ($P < 0.05$). [6] Increased by abomasal infusion, ($P < 0.01$).

infusions will increase milk protein synthesis and/or milk yield. However, to date, mixtures of amino acids have not been infused into the abomasum of lactating cows.

No significant differences in nitrogen intake or nitrogen infused were observed when sodium caseinate was infused into the abomasum or into the rumen (table 2). Cuthbertson and Chalmers (14) have suggested that entry of high quality protein into the rumen may not be as beneficial to the host as entry into the abomasum. In support of this concept our study showed that fecal and urinary nitrogen (g/day), fecal and urinary nitrogen as a percentage of nitrogen intake and urinary nitrogen as a percentage of apparent absorbed nitrogen were significantly decreased when sodium caseinate was administered per abomasum. Milk nitrogen, nitrogen retained and productive nitrogen (milk nitrogen + nitrogen retained) expressed as grams per day and as percentage of nitrogen intake increased when sodium caseinate was infused postruminally. Nitrogen retained and productive nitrogen as a percentage of absorbed nitrogen significantly increased with abomasal administration of sodium caseinate while no significant difference was observed in milk nitrogen as a percentage of absorbed nitrogen. Similar findings have been reported (12, 15–17) [3, 11] when casein and other proteins were administered postruminally into steers, sheep and lactating cows.

Jugular plasma levels of lysine, histidine, isoleucine, leucine, threonine, tyrosine, phenylalanine, alanine and serine were significantly elevated when sodium caseinate was infused postruminally (table 3). Jugular plasma levels of arginine, valine, methionine, cystine, aspartic acid, glutamic acid and glycine were not significantly changed due to site of sodium caseinate infusion. Plasma levels of arginine, threonine, and phenylalanine as a percentage of essential amino acids were significantly depressed while leucine and valine levels were significantly increased when sodium caseinate was infused per abomasum. Jugular plasma glucose and urea concentrations were not significantly different when sodium casein-

[11] Hale, G. D. & Jacobson, D. R. (1972) Feeding or abomasal administration of casein, gelatin, partially delactosed whey (PDW), or zein to lactating cows. J. Dairy Sci. *55*, 709 (abstr.).

TABLE 4

Estimated amino acid and glucose extraction[1] and amino acid secretion in milk by the lactating bovine mammary gland for a 24-hour period

Variable	Site of sodium caseinate infusion: Rumen Uptake	Rumen Extraction[2]	Rumen Output	Abomasum Uptake	Abomasum Extraction[2]	Abomasum Output	Rumen Uptake	Rumen Extraction[2]	Rumen Output
	g	%	g	g	%	g	g	%	g
Lysine[4]	54.6 ± 3.6[3]	63.1± 3.1	52.9± 5.0	102.5 ± 13.7	63.9± 4.0	60.3± 5.8	79.6 ± 22.5	61.6±7.8	54.6± 6.3
Histidine[4]	20.8 ± 3.6	30.2± 3.0	17.6± 1.7	27.2 ± 7.9	24.3± 5.2	20.1± 1.9	33.1 ± 8.1	32.6±3.7	18.2± 2.1
Arginine[4]	57.8 ± 10.6	51.6± 1.9	22.9± 2.2	91.8 ± 16.5	55.2± 4.9	26.1± 2.5	84.5 ± 21.5	48.0±9.2	23.6± 2.7
Isoleucine[4,5]	49.3 ± 4.6	45.8± 2.5	38.5± 3.6	92.6 ± 18.0	50.3± 2.0	43.9± 4.2	67.9 ± 16.0	48.4±5.6	39.8± 4.6
Leucine[4,5]	73.8 ± 8.2	46.1± 3.5	60.4± 3.1	132.7 ± 24.3	48.9± 1.7	72.2± 6.9	95.6 ± 26.0	48.7±7.8	65.4± 7.5
Threonine[4,6]	32.3 ± 4.5	30.2± 2.4	30.1± 2.8	54.3 ± 11.6	37.8± 2.2	34.2± 3.3	51.8 ± 11.4	35.8±3.4	30.9± 3.6
Valine[4]	76.6 ± 22.0	32.7± 3.7	43.1± 4.1	117.6 ± 28.1	28.9± 1.7	49.1± 4.7	94.2 ± 18.0	33.7±3.5	44.5± 5.1
Methionine[4,6]	16.4 ± 1.8	57.0± 5.2	17.0± 1.6	26.4 ± 3.8	67.5± 4.6	19.4± 1.9	24.4 ± 4.4	57.2±8.3	17.5± 2.0
Cystine	−0.7 ± 1.1		4.5± 0.9	−3.7 ± 5.0		6.0± 0.6	3.6 ± 3.8		5.4± 0.6
Phenylalanine[4,5]	22.5 ± 3.6	37.8± 4.3	32.0± 3.0	40.7 ± 6.7	50.7± 2.7	36.5± 3.5	36.5 ± 6.5	49.7±6.0	33.0± 3.8
Tyrosine[4]	26.1 ± 3.5	40.5± 4.5	33.3± 3.1	47.9 ± 8.5	48.8± 2.0	36.6± 3.8	42.5 ± 10.4	47.2±7.7	35.1± 4.0
Alanine[4]	20.9 ± 6.4	11.9± 2.5	22.2± 2.1	16.5 ± 9.1	10.2± 3.6	25.3± 2.4	36.8 ± 10.6	17.0±2.7	22.9± 2.6
Aspartic acid[4]	3.8 ± 2.7	11.6±25.0	51.6± 4.9	5.1 ± 2.0	23.4±11.7	58.5± 5.6	4.2 ± 1.5	29.0±8.6	44.6±10.8
Glutamic acid[4]	58.6 ± 10.1	68.2± 2.8	143.0±13.4	46.3 ± 10.2	51.7± 5.9	163.0±15.6	55.4 ± 9.3	63.9±7.8	147.7±16.9
Glycine[4]	7.8 ± 5.8	3.1± 2.6	13.1± 1.2	27.3 ± 10.1	3.1± 3.0	14.9± 1.4	25.0 ± 4.0	12.0±2.6	13.5± 1.6
Serine[4]	26.0 ± 3.4	32.7± 6.7	36.6± 3.5	43.0 ± 7.5	41.8± 4.4	41.7± 4.0	46.8 ± 11.1	46.4±7.2	37.8± 4.3
Urea	0.15± 0.00	6.7± 1.8		0.30± 0.13	10.6± 3.6		0.10± 0.04	5.6±1.7	
Glucose[5]	2255.6 ±221.7	28.7± 2.7		2989.2 ±482.1	33.2± 2.3		1903.6 ±156.8	28.6±2.4	

[1] Corrected for changes in plasma concentration due to hemodilution using hematocrit values. [2] $\left(\frac{1.0 - \text{concentration of the amino acid or glucose in mammary venous plasma}}{\text{concentration of the amino acid or glucose in arterial plasma}}\right) \times 100$. [3] Values are mean of six cows ±SEM. [4] Output increased by abomasal infusion ($P < 0.01$). [5] Uptake increased by abomasal infusion ($P < 0.05$). [6] Percentage extraction increased by abomasal infusion ($P < 0.05$).

TABLE 5

Estimated jugular response due to abomasal infusion of sodium caseinate

Variable	Increase in jugular plasma amino acid concentration due to sodium caseinate infusion into abomasum vs. into the rumen	Amino acid composition of sodium caseinate	Increase in plasma amino acid concentration / % composition of amino acid in sodium caseinate	
	mg/100 ml	%		
Lysine	0.387±0.039[1]	14.00	0.0276±0.0060[2] (5)[3]	0.3154 (5)[4]
Histidine	0.269±0.045	5.25	0.0512±0.0090 (6)	0.4685 (7)
Arginine	0.183±0.091	6.74	0.0272±0.0130 (4)	0.0682 (2)
Isoleucine	0.783±0.319	10.47	0.0748±0.0300 (7)	0.4087 (6)
Leucine	1.217±0.439	16.16	0.0753±0.0270 (8)	0.5136 (8)
Threonine	0.126±0.040	8.57	0.0147±0.0047 (1)	−0.2917 (1)
Valine	1.381±0.182	13.43	0.1028±0.0135 (9)	1.0424 (9)
Methionine	0.128±0.080	5.00	0.0256±0.0160 (3)	0.2240 (4)
Cystine	−0.053	0.53		
Phenylalanine	0.132±0.033	8.8	0.0150±0.0037 (2)	0.0840 (3)
Tyrosine	0.191±0.034	11.12	0.0170±0.0035	

[1] Values represent the mean±SEM, seven cows. [2] Present study. [3] Numbers in parentheses indicate a possible limiting sequence of amino acids. [4] Values calculated using data of Broderick et al. (4).

ate was infused into the rumen versus into the abomasum.

Levels of the other essential amino acids as a percentage of the total essential amino acids were not significantly affected by the site of sodium caseinate infusion. Total essential amino acids, excluding tryptophan, as a percentage of total amino acids assayed were significantly increased when sodium caseinate was infused into the abomasum. These data and other reports suggest that plasma amino acid levels are markedly affected by dietary nitrogen and amino acid or protein infusions into the abomasum (1, 3, 4, 18–20).[12]

Estimated extraction by the mammary gland of cystine, alanine and glutamic acid per 24 hours was decreased; however, all other amino acids were extracted in larger quantities when casein was infused postruminally (table 4). The increased extraction during the abomasal infusion was significant for only isoleucine, leucine and phenylalanine. In general, the greater uptake of amino acids and the larger standard error of the mean for the last rumen infusion period as compared with the first rumen infusion period can be attributed to one cow which extracted extremely large quantities of amino acids. During the first period of sodium caseinate infusion into the rumen, estimated amino acid uptake by the gland was sufficient to account for all of the essential amino acids secreted in milk except for phenylalanine and methionine. Other investigators (21–23) have shown a deficiency in nonessential amino acids extracted as compared with amino acids secreted in milk. However, it has been suggested that nonessential amino acids can be synthesized by the lactating mammary gland (2, 21–24).

Percentage extraction by the mammary gland of threonine and methionine increased significantly, and arginine, phenylalanine and tyrosine were increased but not significantly (table 4). The percentage extraction of histidine, isoleucine, valine, alanine and glutamic acid decreased when sodium caseinate was infused postruminally. Site of sodium caseinate infusion did not significantly influence percentage extraction of glucose or urea by the mammary gland; but the total quantity of glucose extracted was significantly increased.

It has been suggested that methionine may be the most limiting amino acid for milk production in the lactating dairy cow. Studies (5, 6) have shown milk production to be increased when methionine hydroxy analog was fed. However, no significant increase in feed intake, milk yield or milk protein has been observed in other studies when methionine was treated to bypass the

[12] Poley, G. E. & Trenkle, A. H. (1962) Influence of nitrogen source on amino acid patterns in plasma and abomasal ingesta from sheep. J. Anim. Sci. *22*, 1139 (abstr.).

TABLE 6

Average daily actual and theoretical milk protein[1] output and ratio of amino acid uptake by the gland to output in milk

Variable	Site of sodium caseinate infusion		Ratio: Uptake from plasma / Output in milk	
	Abomasum	Avg of rumen infusions	*During abomasum infusions*	*During rumen infusions*
Actual, g/day	785±77[2]	715±108		
Theoretical, g/day				
Lysine	1289±364 (5)[3]	887± 3.19 (3)	1.72 (5)	1.24 (3)
Histidine	1027±300 (2)	1021±364 (7)	1.35 (2)	1.50 (7)
Arginine	2469±574 (9)	2099±683 (9)	3.54 (9)	3.03 (9)
Isoleucine	1601±313 (7)	1021±331 (6)	2.11 (7)	1.48 (6)
Leucine	1397±255 (6)	938±300 (5)	1.85 (6)	1.33 (5)
Threonine	1206±255 (4)	931±306 (4)	1.59 (4)	1.32 (4)
Valine	1818±434 (8)	1333±453 (8)	2.41 (8)	1.93 (8)
Methionine	1040±147 (3)	766±204 (2)	1.37 (3)	1.12 (2)
Phenylalanine	849±141 (1)	632±185 (1)	1.11 (1)	0.91 (1)
Tyrosine	957±172	766±230		

[1] Theoretical yield of milk protein based on the uptake of the specific amino acid. [2] Values are mean of six cows ±SEM. [3] Numbers in parentheses indicate the order of limiting essential amino acids.

rumen (4) or infused intravenously.[13, 14] Since methionine may be no more limiting than several other amino acids (1), this may explain the difference in the above research reports.

Jacobson et al. (12) indicated lysine, methionine, threonine, and histidine as the order of limiting amino acids for cows fed a low sulfur diet; however, the sequence for cows fed sulfur-supplemented diets appeared to be lysine, methionine, histidine, and threonine. Chandler[15] calculated from the amino acid composition of microbial protein that methionine, valine, isoleucine, tryptophan, and lysine limited milk production. Virtanen (25) observed low jugular plasma levels of histidine and methionine when purified diets containing urea were fed to lactating cows. Brown[16] using the data of Virtanen (25), calculated that methionine and histidine were the two most limiting amino acids for milk production.

Data in tables 5 and 6 were calculated to determine a possible sequence of limiting amino acids for milk protein synthesis. When an amino acid or protein is infused into the abomasum, the increase in plasma concentration of the essential amino acid(s) should be proportional to the quantity of amino acid(s) infused if the amino acid(s) is not limiting body functions (4). Hence, if the essential amino acid(s) infused into the abomasum is limiting, the increase in plasma concentration relative to the amount infused should be smaller when compared to amino acids that are not limiting. Therefore, in the present study the increase in jugular plasma concentration of each essential amino acid during sodium caseinate infusion into the abomasum was compared with the amino acid composition of the infused casein (26, 27). Using this method the sequence of limiting essential amino acids was threonine, phenylalanine, methionine, arginine, lysine, histidine, isoleucine, leucine and valine (table 5). Similar calculations using the data of Broderick et al. (4) showed that the limiting sequence of amino acids was threonine, arginine, phenylalanine, methionine, lysine, isoleucine, histidine, leucine, and valine (table 5).

The theoretical quantity of milk protein which could be synthesized from the uptake of each amino acid is shown in table 6. The amino acid with the lowest theoretical output of protein was considered to be the most limiting for milk protein synthesis.

[13] Fisher, L. J. (1969) Effect of methionine infusion on milk production and plasma free amino acids of lactating cows. J. Dairy Sci. *52*, 943 (abstr.).

[14] Teichman, R., Carvolo, E. V. & Mochrie, R. D. (1969) Milk production and composition responses to intravenous infusion of L-methionine. J. Dairy Sci. *52*, 942 (abstr.).

[15] Chandler, P. T. (1970) Improving protein nutrition of ruminants. Proceedings 1970 Virginia Feed Convention and Nutrition Conference, pp. 1–8.

[16] Brown, R. E. (1969) The conversion of nutrients into milk. The University of Nottingham Third Nutrition Conference for Feed Manufacturers, pp. 23–42.

The average theoretical protein synthesis for the two rumen infusion periods indicated the sequence of limiting essential amino acids to be phenylalanine, methionine, lysine, threonine, leucine, isoleucine, histidine, valine, and arginine (table 6). The sequence of limiting essential amino acids was essentially the same when sodium caseinate was infused into the abomasum. The most significant change occurred with histidine which was seventh most limiting in the sequence during rumen infusion periods and second most limiting during the abomasal infusion period. The percentage extraction of histidine by the mammary gland during sodium caseinate infusion into the abomasum was decreased by about 7%. The ratio of amino acids extracted to amino acids secreted in milk also is shown in table 6.

A comparison of the sequence of limiting amino acids obtained, using the jugular plasma concentrations and the arteriovenous uptake of amino acids during the rumen infusion periods, shows that they are similar with the exception of threonine and arginine. No explanation for the difference in threonine can be given at this time. Since the jugular plasma concentrations indicate arginine to be limiting for the lactating cow and the arteriovenous difference shows the uptake of arginine to be more than adequate for milk protein synthesis, this suggests that arginine may have a requirement in the mammary gland other than for milk protein synthesis. Other reports (21, 23), in contrast to our finding, show that the uptake of arginine by the lactating bovine mammary gland was just sufficient for milk protein synthesis. Mepham and Linzell (23) also reported that in the goat but not in the cow, arginine was extracted by the mammary gland in excess of the requirement for milk protein synthesis and suggested the excess arginine was used for synthesis of nonessential amino acids. Ornithine also was reported to be used by the gland for synthesis of nonessential amino acids. Uptake of arginine and valine by the lactating sow mammary gland also has been reported in excess of that required for milk protein synthesis (22). Even though Barry (28), Linzell (2) and Mepham and Linzell (23) have discussed limitations of the arteriovenous technique, it appears to offer a method for elucidating patterns of amino acids which may be limiting milk yield and/or milk protein synthesis.

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Food Protection Committee 1972

Total Annual Poundage --- Sodium Caseinate

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table II, Part A, p. 10

Food Protection Committee 1972

Usage Levels --- Sodium Caseinate

Nat. Acad. Sci., Nat. Res. Coun.
Table 2, pp. 34-35

Food Protection Committee 1972

Comprehensive GRAS Survey, Daily Intakes --- Sodium Caseinate

Nat. Acad. Sci., Nat. Res. Coun.
Table 14, Part A pp. 57, 67, 77, 102, 112, 158-159,
171, 184, 194, 202-203, 216, 246, 256, 281

Food Protection Committee 1972

Comprehensive GRAS Survey, Daily Intakes—Casein, Calcium Caseinate

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table 15, p. 19

Food Protection Committee 1972

Comprehensive GRAS Survey, Daily Intakes -- Sodium Caseinate

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table 13, Part A 168-170

I. Oral Challenge with Milk and Isolated Milk Proteins in Allergic Children

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MANY STUDIES have indicated that respiratory, dermal, gastrointestinal, anaphylactic, and other reactions may result from allergy to cow's milk.[1-3] However, considerable doubt of the diagnosis of milk allergy has been expressed by a large segment of the medical profession because of the paucity of objective evidence. Most reported cases have been accepted on the basis of symptomatic improvement following milk elimination. Some reports of milk allergy seem convincing because they are based upon symptoms produced by feeding trials as well as improvement following milk elimination, but such observations have been limited to single feedings of cow's milk in occasional patients with severe manifestations, while few corollary immunologic studies have been made.

Recently, a syndrome of recurrent pulmonary disease, iron-deficiency anemia, poor growth, and gastrointestinal symptoms have been related to milk allergy.[4-6] In these cases precipitin antibodies to milk were found, symptomatic improvement followed milk elimination, and recurrence of symptoms followed reintroduction of milk. Oral challenge studies[7] and the finding of milk antibodies[8] have implicated milk allergy in some patients with ulcerative colitis. Gunther *et al.*[9] found high anti-milk protein hemagglutinin titers in normal human infants. Antibodies to two constituents of cow's milk, bovine serum albumin (BSA) and alpha-lactalbumin, have been found in many normal adults and children without evidence of milk allergy. Other milk antigen-antibody systems have not been well defined as they relate to healthy individuals. Parish *et al.*[11] suggested that sudden, unexpected deaths of hypersensitive children with high anti-milk hemagglutinin titers may be caused by aspiration of milk.

It is clear even from this brief review that more definitive studies of milk allergy are indicated to answer many of the clinical and immunologic questions which have been posed. An additional impetus for such studies has been the development of techniques for isolating certain major milk proteins in sufficient quantity and purity for

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use in human feeding experiments and immunochemical investigations.

Investigations with sensitized animals have shown the relative antigenicity of milk proteins.[11-14] However, there are no studies concerning allergic properties of the milk proteins based upon challenge feedings of naturally sensitized humans. Such information would be germane to the understanding and therapy of patients allergic to milk.

The purposes of this study were to determine the symptoms of milk allergy by repeated oral challenges with milk and/or milk proteins, to determine the relative allergenicity of each of the isolated milk proteins in children allergic to milk and to document the clinical course of children with milk allergy.

MATERIALS AND METHODS

In December, 1960, representatives of the pharmaceutical division of The Borden Company (D.W.A. and S.S.) and 17 pediatricians and pediatric allergists from Arkansas, Louisiana, Tennessee, and Texas, met and agreed upon a protocol. The cardinal point of the investigation, the basis of patient selection, was delineated carefully. A diagnosis of milk allergy was accepted only when (1) symptoms subsided following milk elimination; (2) symptoms occurred within 48 hours following a trial feeding of milk;* (3) three such challenges were positive and similar as to onset, duration and clinical features; (4) symptoms subsided following each challenge reaction. The details of the protocol follow.

Initially, the patient's symptoms, allergy history, dietary history, family history, and physical findings were recorded. If milk sensitivity was suspected and permission to study the patient was obtained, blood was collected for serologic studies, skin testing with milk proteins was done, and a milk elimination diet trial was begun. This diet usually was restricted to the foods shown in Table I, and to a vitamin supplemented soybean milk (Mull-Soy, supplied by The Borden Company). In three cases, hydrolyzed casein milk or meat base milk was used.

Oral Challenge Studies

If the symptoms subsided promptly upon milk elimination, the patient returned to the physician's office and a physical examination was performed to confirm improvement and to serve as a base line for provocative tests. Then, 100 ml of skim milk was offered to the patient. The amount of milk consumed, time of onset and duration of reactions, and the signs, symptoms, and severity of the challenge response were recorded. Symptoms beginning after the patient left the office (usually 3-4 hours) were reported by the parent and confirmed by a subsequent physical examination. Following each positive oral challenge, milk elimination was reinstituted.

If a reaction failed to occur following a single 100-ml dose of skim milk, larger amounts of cow's milk were added to the diet without addition of other "new" foods to the basic diet. If no symptoms were

TABLE I

APPROVED FOODS LIST FOR MILK-FREE DIET

Category	*Specific Foods*
Meats	Lamb, pork, bacon
Cereals	Rice, rice flour, rye and rye flour
Fruits	Pineapple, apple, banana
Vegetables	Irish potato, sweet potato, lettuce, carrots, asparagus, English peas
Fats and Oils	Soy oil, sesame oil, Willow Run oleomargarine (milk-free)
Miscellaneous	Knox sparkling gelatin, plain with following flavors: tapioca, vanilla, molasses, sugar, salt

* This time interval was chosen because it was felt that all patients could be followed carefully for such a period to determine the onset time. There is evidence that symptoms from some types of cow's milk hypersensitivities may not be manifest clinically for weeks or months.

produced, the patient was excluded from the study. If a reaction occurred to larger amounts of milk, similar feeding trials were repeated after periods of milk elimination. Milk allergy was accepted if reactions produced by three oral challenges were extremely similar and improvement occurred following each milk elimination.

Whenever possible, those patients who reacted to 100 ml skim milk doses were challenged orally to each of four milk proteins, casein, bovine serum albumin (BSA), beta-lactoglobulin, and alpha-lactalbumin. Usually several days intervened between oral challenges. If the last oral protein challenge was negative, a repeat skim milk challenge was done to insure that the characteristics of the challenge reaction were unchanged. Following the last oral challenge, milk elimination was reinstituted and continued until the patient was found to be unreactive by trial milk feedings.

Preparation of Milk and Milk Proteins

The milk challenge, supplied prepackaged, consisted of 9 gm of a low-heat, nonfat dry milk,* mixed with 4 gm of sucrose. This milk was obtained from a uniform lot of a commercial skim milk preparation (Starlac). The amount of euglobulins and pseudoglobulins in this skim milk is somewhat less than is found in raw or homogenized milk. Otherwise, the protein constituents appeared to be very similar.

The milk proteins, casein, beta-lactoglobulin, alpha-lactalbumin and BSA were selected for oral challenges because methods for their isolation and purification were available.

Casein was prepared by repeated solubilization and precipitation as previously described.[14] Contamination of the casein with whey proteins was estimated to be 2% or less as measured by electrophoresis on paper or cellulose acetate.

* 36.7% protein, 51% lactose, 0.87% fat, 8.0% minerals, 3.5% moisture; coliform negative; standard plate count 890 per gram, meeting U.S. Public Health standards for grade A milk.

TABLE II

AMOUNT OF CASEIN, BETA-LACTOGLOBULIN, ALPHA-LACTALBUMIN AND SERUM ALBUMIN IN 100 ML OF FLUID SKIMMED COW'S MILK

Protein	*Amount (mg/100 ml)*
Casein	2,800
Beta-lactoglobulin	420
Alpha-lactalbumin	70
Serum albumin (BSA)	20

Beta-lactoglobulin was prepared by the methods of Larson and Jenness[15] and Aschaffenburg and Drewry.[16] The protein was crystallized at least six times and showed no alpha-lactalbumin when tested immunologically by agar gel diffusion.

Alpha-lactalbumin was prepared by the method of Gordon and Ziegler.[17] The protein was recrystallized at least four times or until no beta-lactoglobulin could be detected by agar gel diffusion against a rabbit serum specific for beta-lactoglobulin. Contamination with BSA was estimated to be 2% or less by means of electrophoresis on cellulose acetate in barbital buffer pH 8.6μ = 0.05.

Bovine serum albumin was obtained from the Armour Company, Kankakee, Illinois. Contamination of BSA with gamma globulin was estimated by paper electrophoresis to be less than 3%.

Each protein challenge, supplied prepackaged, consisted of the quantity of that protein in 100 ml of skim milk (Table II), mixed dry with 4 gm of sucrose. Immediately prior to use, the powdered skim milk and protein challenges were reconstituted with 100 ml tap water.

At periodic intervals and at the end of the study, three investigators reviewed the patient records. Patients were designated allergic to milk only when sufficient data was present to satisfy the previously stated criteria.

Statistical analyses were done by the University of Texas Medical Center Research Computation Center, Galveston, Texas.

TABLE III

DISTRIBUTION OF AGE OF ONSET OF SYMPTOMS OF MILK ALLERGY IN 89 MILK ALLERGIC PATIENTS

Age	Group A Patients*		Group B Patients*	
	No.	%	No.	%
<1 mo	28	62	24	55
1–2 mo	8	18	3	7
2–3 mo	5	11	4	9
3–6 mo	2	5	5	11
6–12 mo	0	0	2	4.5
12–24 mo	0	0	2	4.5
2–4 yr	1	2	0	0
4–7 yr	0	0	2	4.5
11 yr	0	0	2	4.5
Unknown	1	2	0	0

* Group A were challenged orally with milk and purified milk proteins. Group B were challenged orally with milk only.

RESULTS

Approximately 700 pediatric patients suspected of milk allergy were examined during the course of this study. Of these, 150 case records were submitted by the clinical investigators. Eighty-nine of the case records were accepted as satisfying the criteria originally established for the diagnosis of milk allergy.

To facilitate analysis and presentation of the data, the 45 patients who were challenged with both milk and the individual milk proteins were designated as Group A, and the 44 who were challenged with milk only were designated as Group B.

One patient in Group A was tested with amounts of purified proteins found in 200 ml of skim milk, since she reacted to that amount and not to the 100-ml dose. Sixteen patients in Group B reacted to 100 ml skim milk dosage, but were not tested further because of the severity of the reactions, or the patient was otherwise unavailable for further challenges. The remaining 28 Group B patients reacted only to larger amounts of milk, and thereby were ineligible for challenge with the proteins.

For Group A, the median age studied was 6 months with a range between 2 weeks and 6 years; the median age of onset was 1 month with a range between 1 week and 3.5 years; the median duration of the milk allergy was 12 months with a range of 8 weeks to 7 years. For Group B, the median age studied was 10 months with a range of 6 weeks to 13 years; the median age of onset was 1 month with a range of one week to 8 years; the median duration of milk allergy was 13 months with a range of 6 weeks to 8 years. The distribution of the ages of onset of allergic symptoms is listed in more detail in Table III.

In Group A, 30 patients (67%) were on a heat processed milk* diet, 5 (11%) were taking a heat processed milk in combination with breast milk, 3 (7%) were on a heat processed milk in combination with homogenized milk, 3 (7%) were on homogenized plus breast milk and 1 (2%) was on homogenized milk. The original milk diet could not be determined for 3 patients. In Group B, 24 (55%) patients were on a heat processed milk, 7 (16%) were taking a heat processed milk in combination with breast milk, 5 (11%) were on a heat processed milk in combination with homogenized milk, 2 (5%) were on homogenized plus breast milk and 2 (5%) were on homogenized milk. The prior milk diet could not be determined for 4 patients.

A positive family history of allergy (chronic asthma, rhinitis, urticaria, or dermatitis) occurred in 80% of Group A patients, and in 73% of Group B patients. Four relatives of Group A patients and five of Group B patients were said to be allergic to milk but were unavailable for examinations.

Symptomatology

The presenting and challenge symptoms of each patient are detailed in Appendix A and Appendix B. The frequency of each presenting and challenge symptom in the two groups were compared (Table IV) and found to be quite similar. Multiple symp-

* Heat processed milks included evaporated milk, Bremil Liquid, Enfamil Liquid, Similac Liquid, etc.

TABLE IV

INCIDENCE OF PRESENTING SYMPTOMS AND SYMPTOMS UPON ORAL CHALLENGE WITH MILK

Symptom	Group A Patients*				Group B Patients*			
	Presenting		Challenge		Presenting		Challenge	
	No.	%	No.	%	No.	%	No.	%
Rhinitis	19	43	15	34	22	50	16	36
Asthma	16	37	15	34	12	27	9	20
Atopic dermatitis	18	41	14	32	19	43	17	39
Vomiting	15	34	17	37	9	20	12	27
Abdominal pain	18	41	18	41	10	23	7	16
Diarrhea	21	47	19	43	15	34	14	32
Urticaria	4	10	6	13	3	7	4	9
Anaphylaxis	2	5	4	10	2	4.5	4	9
Central nervous system and others	3	6	4	10	9	17	12	27

* Group A were challenged orally with milk and purified milk proteins. Group B were challenged orally with milk only.

toms were produced by challenges in 77% of both Group A and Group B patients. The challenge symptoms were as follows.

Vomiting occurred during challenge reactions in 29 patients (33%). Gagging, spitting, or emesis generally began within the first hour following oral challenge. Vomiting was never projectile or prolonged.

Diarrhea occurred during challenge reactions in 33 patients (37%). These stools were usually loose and contained mucus. The diarrheal symptoms rarely persisted more than a day. Two patients with diarrhea had bloody stools as a consequence of every challenge. Steatorrhea occurred in one patient.

Abdominal pain or *colic* occurred during challenge reactions in 25 patients (28%). Colic was characterized in infants by excessive prolonged crying, often with flexion of the thighs upon the abdomen and followed by exhaustion. Abdominal pain in older children was usually peri-umbilical or poorly localized. These gastrointestinal symptoms were frequently present together (Case 1, Appendix D). Vomiting or diarrhea resulted in dehydration in only two patients.

Rhinitis occurred during challenge reactions in 31 patients (35%). Nasal obstruction due to mucosal edema and serous or mucoid secretions was the most frequent manifestation (Case 2, Appendix D). Sneezing and nasal pruritus were less frequent.

Asthma occurred during challenge reactions in 24 patients (27%). The principal finding was expiratory sibilant rales. Inspiratory wheezes were also frequent. In three patients the wheezing was found only with aid of the stethoscope. Early in the reaction, a few patients had crepitant rales or subcrepitant rales (Cases 2 & 3, Appendix D). In four patients with rhinitis or asthma, signs of a secondary respiratory tract infection (fever, otitis media, pneumonitis) were produced following the early stages of challenge reaction.

Atopic dermatitis occurred during challenge reactions in 31 patients (35%). Usually the early stages of the dermatitis, minute erythema or papules, constituted the reaction (Cases 4 & 5, Appendix D). Vesicles did appear in one patient. The dermal lesions were usually pruritic and found at previously involved sites on the cheeks or flexor surfaces of the extremities, but frequently occurred on the back, abdomen, and thighs. Many reactions lasted 24 to 72 hours. Frequently some residual lichenification persisted between challenges. Six pa-

TABLE V

REPRODUCTION OF PRESENTING SYMPTOMS BY ORAL CHALLENGE WITH MILK

Symptom	Percentage of Patients Showing Recurrence of Presenting Symptom Upon Oral Challenge	
	Group A*	Group B*
Allergic rhinitis	71	68
Bronchial asthma	75	75
Atopic dermatitis	76	89
Urticaria	100	67
Anaphylaxis	100	100
Vomiting	69	78
Diarrhea	70	80
Abdominal pain	80	60
CNS	100	90

* Group A were challenged orally with milk and purified milk proteins. Group B were challenged orally with milk only.

tients, two in Group A and four in Group B, had only atopic dermatitis by history and milk challenge.

Urticaria occurred during challenge reactions in 10 patients (11%). Wheals, angioedema, or large areas of erythema appeared following oral challenge within a few minutes to an hour (Case 5, Appendix D).

Anaphylaxis occurred during challenge reactions in eight patients (9%). Only a small amount of milk was needed to produce sudden alarming reactions with profound weakness and seizures or signs of shock. In both Group A and Group B, the four patients with a history of generalized urticaria had anaphylactic reactions following milk or protein challenges (Case 5, Appendix D). However, three children in Group B who had anaphylaxis did not have urticaria as a presenting or challenge symptom. One of these had tonic-clonic seizures, and diarrhea following milk ingestion (Case 6, Appendix D).

Central Nervous System or Other Symptoms occurred in 16 patients (18%). These were lethargy, weakness, irritability, excessive crying, restlessness, prolonged pallor, or infra-orbital darkening. Patients with these symptoms alone were not included in this study.

The presenting symptoms were reproduced by challenge feedings in 79% of Group A and 77% of Group B patients. The comparative reproducibility for each symptom is listed in Table V. Challenge feedings produced symptoms not found in the history in seven Group A patients and in nine Group B patients (Table VI). Vomiting was the most frequent additional symptom produced.

A listing of various symptom combinations was made, and no consistent pattern was found.

The onset and duration of challenge reactions are presented in Tables VII and VIII respectively. In Group A, the median reaction onset time was 1 hour, with a range between 10 seconds and 25 hours; the median reaction duration was 24 hours, with a range of 40 minutes to 96 hours. In Group B, the median reaction onset time was 12 hours, with a range of 1 minute to 7 days; the median reaction duration was 36 hours, with a range of 30 minutes to 15 days.

In Group B, the median reaction onset time in those who reacted to 100 ml milk challenges was 2 hours, but the median reaction onset time in those who required larger amounts of milk to elicit reactions

TABLE VI

SYMPTOMS PRODUCED BY MILK CHALLENGES, NOT PRESENT IN THE HISTORY OF THE MILK ALLERGIC PATIENT

Symptoms	Number of Patients	
	Group A*	Group B*
Allergic rhinitis	1	1
Bronchial asthma	2	0
Atopic dermatitis	1	0
Urticaria	2	2
Anaphylaxis	2	2
Vomiting	6	5
Diarrhea	4	2
Abdominal pain	1	1
CNS	1	3

* Group A were challenged orally with milk and purified milk proteins. Group B were challenged orally with milk only.

was 24 hours. No significant difference in reaction duration in these two divisions of Group B patients was found.

When challenged orally, every patient in Group A evidenced an allergic reaction to one or more of the purified proteins. The reaction by patient is shown in Appendix C. The incidence of reactions to these proteins (Table IX), were casein, 60%; BSA, 52%; beta-lactoglobulin, 62%; and alpha-lactalbumin, 53%. Nineteen reacted to one protein, 12 to 2 proteins, 7 to 3 proteins, and 6 to all 4 proteins. Further attempts to quantitate the minimal amount of protein necessary to produce an allergic response were carried out in two patients. In the first, 7.5 mg of alpha-lactalbumin caused wheezing. In the second, less than one microgram of casein, beta-lactoglobulin or alpha-lactalbumin produced anaphylaxis. This same patient was insensitive to 20 mg and 40 mg challenges with BSA.

In a given patient, the oral challenge reactions to skim milk or purified milk proteins were similar with respect to time of onset, type, intensity and duration of symptoms. In patients responding to more than one protein, a similar likeness of reactions was observed.

Four patients of Group A (9%) and 12 of Group B (27%) experienced spontaneous remissions. In these patients, the duration of milk allergy, as calculated from the onset of symptoms to the date that milk was tolerated in the diet, varied from 8 weeks to 7 years with a median of 7 months.

TABLE VII

TIME OF ONSET OF ALLERGIC SYMPTOMS AFTER ORAL CHALLENGES WITH MILK OR PURIFIED MILK PROTEINS

Onset Time after Milk Consumption	*Group A* Patients*		*Group B* Patients*	
	No.	*%*	*No.*	*%*
<6 min	12	27	4	9
6–30 min	10	22	5	11
30–60 min	3	7	1	2
1–3 hr	8	18	3	7
3–6 hr	5	11	3	7
6–12 hr	4	9	5	11
12–24 hr	3	6	12	27
1–3 da	0	0	10	23
7 da	0	0	1	2

* Group A were challenged orally with milk and purified milk proteins. Group B were challenged orally with milk only.

TABLE VIII

DURATION OF SYMPTOMS RESULTING FROM ORAL CHALLENGES WITH MILK OR PURIFIED MILK PROTEINS

Duration of Symptoms	*Group A* Patients*		*Group B* Patients*	
	No.	*%*	*No.*	*%*
0–30 min	0	..	3	7
30–60 min	5	11	0	..
1–2 hr	1	2	0	..
2–4 hr	1	2	0	..
4–6 hr	0	..	2	4.5
6–12 hr	4	9	1	2
12–24 hr	16	35	9	20
24–48 hr	5	11	14	32
2–4 da	13	30	12	27
4–7 da	0	..	2	4.5
7–14 da	0	..	1	2

* Group A were challenged orally with milk and purified milk proteins. Group B were challenged orally with milk only.

Correlation coefficients were calculated for all combinations of the following categories: presenting and challenge symptoms, age studied, age of onset of allergic disease, duration of hypersensitivity, onset of reaction after challenge, reaction severity, reaction duration, amount of milk required to produce reactions ,and particular proteins which produced reactions. The only significant correlations found were the relationship of the onset of reaction following oral challenge to the amount of antigen required to produce the reaction, and the relationship of the symptom of urticaria to anaphylaxis following oral challenge.

Nine patients in Group A and 5 in Group B had other hypersensitivities, usually to food such as eggs, beef, corn, wheat, oats, and peanuts.

COMMENT

In previous reports, lack of repeated oral

TABLE IX

RESULTS OF ORAL CHALLENGES WITH ISOLATED MILK PROTEINS

Proteins	Number of Patients* Challenged	Number Positive	Per Cent Positive
Casein	45	27	60
Serum albumin (BSA)	44	23	52
Beta-lactoglobulin	37	23	62
Alpha-lactalbumin	34	18	53

* Group A patients only.

challenges and other appropriate controls make it difficult to determine if the patients described were allergic to milk. Fortuitous improvement could have occurred following milk elimination because of elimination of an allergenic chemical additive in the milk, elimination of milk fats or sugars to which the patient was intolerant, remission of a non-allergic disorder, elimination of other allergens in the environment, or remission of other hypersensitivity reactions. In this study, the use of trial feedings of milk or milk proteins in which these additives, fats, and sugars were removed or avoided, averted this criticism. Further, in the group tested with purified proteins, the possibility that these reactions were a placebo effect was discarded because the reconstituted milk proteins had no outward resemblance to milk, and many challenges were negative to one or more proteins while positive to others. The reproducibility of presenting symptoms by challenges indicates the validity of this method for proving milk allergy.

The challenge reactions, which ranged from the violence of anaphylaxis to the mild distress of allergic rhinitis, were quite variable as to type and intensity. Multiple symptoms predominated. Indeed, few patients had the same symptom combination. No symptom or physical sign was found to be peculiar to milk allergy.

Because the early stages of atopic dermatitis were produced by oral challenge in a large percentage of patients with a preceding history of this disorder, it is concluded that atopic dermatitis is frequent in infants allergic to milk. The reverse, a high incidence of milk allergy in patients with atopic dermatitis, has not been confirmed.[18]

The production of anaphylaxis in patients presenting with urticaria indicates that considerable caution must be exercised if oral challenges are to be done with such a patient. Equally important was the demonstration that urticaria does not always precede or accompany severe reactions in the milk allergic child.

A few micrograms of antigen may suffice for the provocation in the patient with extreme sensitivity. Even these minute amounts may prove dangerous as the near death of a few patients in this series demonstrated. Several instances of severe reactions or death have been attributed to milk allergy.[13] If the patients in this series who exhibited anaphylactic reactions had died earlier in the course of their illness, they would have been classed as a sudden, unexpected death in infancy. Parish *et al.*[11] have emphasized that some of these deaths may be explained on the basis of hypersensitivity to milk proteins, due to aspiration of milk by infants with high titers of milk hemagglutinins.

In contrast, comparatively large amounts of milk or repeated ingestion of milk were necessary to produce many of the reactions in some of the Group B patients. Also, the physical signs of some reactions, such as erythema, minute papules, nasal mucosal edema, sibilant rales, and other auscultatory findings, would have been missed except for careful medical observation during the challenges.

Asthma and rhinitis are more frequent than previously reported in milk allergic children of this age. Many of these patients had a history of recurrent respiratory infections. Is is important that the pulmonary auscultatory findings in some of these patients were indistinguishable from those in pneumonia and that the nasal symptoms were often mistaken for respiratory infections.

Three patients exhibited recurrent pneumonia, anemia and other features of a syndrome associated with milk allergy as described by Heiner *et al.*[4,5] and Holland *et al.*[6] However, most of our patients did not have the extensive hematologic or radiologic investigations as described in Heiner's or Holland's Groups. This lack of hematologic data also prevented us from determining if iron deficiency anemia was more common in the Group B patients who required larger amounts of antigen to react. This has been evidenced in Heiner's and Holland's studies.

The marked variations in onset, duration and severity of the oral challenge reactions possibly are due to different points of allergen penetration and interaction. Rapid alarming symptoms might result from reactions high in the alimentary or respiratory tracts; slower, less severe symptoms from reactions in the lower intestinal tract. Differences in reaction time in oral and rectal administration of milk antigens in milk allergic subjects, support this possibility.[19] However, many other explanations can be offered. Whether the allergen may have its effects in alimentary tract tissue (localized antibody, sensitized lymphoid cells, or other reacting cells), directly with shock organ tissues or in combination with circulating or tissue fixed antibody is not known.

Recently, Saperstein and Anderson[14] demonstrated that casein, alpha-lactalbumin and beta-lactoglobulin are antigenically active in all heat treated commercial milks and milk formula products and, therefore, could be potential allergens in man. Hypersensitivity to each of the four major milk proteins was found in this study. Eighty per cent of the patients challenged with milk proteins were consuming a heat processed milk prior to and at the time of onset of the allergic symptoms. Also, 87% of the children in Group A gave reactions to challenge doses of the heat stable purified proteins mentioned above (Appendix C).

It appears that the only milk allergic patients which can be treated with a processed type milk are those hypersensitive to BSA or other heat labile proteins. Only 6 of the 45 patients tested were in this category. Of these, only two patients could be managed on a processed milk in which BSA was inactive by laboratory test. The other four patients may have been reactive to one of the proteins not tested, or, as in one case, reactive to a larger amount of protein (casein) than the amount used as the initial challenge. It is possible that other groups of milk allergic children may tolerate the levels of the heat stable proteins, casein, beta-lactoglobulin or alpha-lactalbumin, in processed milks. However, this conjecture should be tested carefully with quantitative oral challenge studies before it is accepted. Evidence found in this study indicates that the treatment of cow's milk allergy should entail complete removal of milk from the diet. Examination of the data in Tables III and IV reveals that children usually become allergic to cow's milk during the first few months of life; which is paradoxically the period of relative immunologic inactivity. During early infancy the diagnosis of milk allergy can be established simply by dietary elimination of cow's milk and foods containing milk. Milk allergy should be strongly suspected when a young infant who is receiving cow's milk develops gastrointestinal, respiratory, dermal, or other symptoms described in this report.

The data concerning the frequency of remission of milk allergy is incomplete because of the short duration of the study. In some patients a considerable period of allergen elimination did not lead to remission since these children were still reactive to milk challenge. Many questions such as the duration of milk allergy, the occurrence of new shock organ responses, and the acquisition of other hypersensitivities are unanswered. We hope to follow some of the patients in this study over a period of years to determine future clinical and immunological events.

The laborious effort, potential dangers to the more sensitive patient, and the quantitative limitations of the oral challenge

(Text continued on page 438)

APPENDIX A

GROUP A. PATIENTS. COMPARISON OF PRESENTING ALLERGIC SYMPTOMS AND SYMPTOMS PRODUCED BY MILK AND PURIFIED MILK PROTEIN CHALLENGES FOLLOWING AN ASYMPTOMATIC PERIOD

Patient	Age of Onset	Presenting Symptoms*									
		Rhin.	Asth.	A.D.	Anap.	Urt.	Vom.	Dia.	Abd. Pain	C.N.S.	Other
1	2 mo	3+	0	0	0	0	2+	2+	0	0	0
2	3 mo	4+	4+	4+	1+	4+	0	0	0	0	0
3	1.5 wk	0	0	0	0	0	0	1+	1+	1+	Perianal dermatitis; blood in stool
4	1 mo	0	0	0	0	0	0	0	1+	0	0
5	1 mo	0	0	3+	0	0	1+	1+	0	0	0
6	2.5 mo	0	0	3+	0	0	0	0	0	0	0
7	2 wk	0	0	0	0	0	0	1+	1+	0	0
8	1 mo	0	0	1+	0	0	0	1+	1+	0	0
9	3 wk	0	0	0	0	0	0	0	1+	0	0
10	2 mo	1+	0	0	0	0	0	1+	1+	0	0
11	6 mo	3+	0	0	0	0	0	1+	1+	0	0
12	3.5 yr	2+	0	0	0	0	0	0	1+	0	0
13	2 wk	0	4+	0	0	0	0	2+	0	0	0
14	2 mo	0	0	1+	0	0	1+	1+	0	0	0
15	1 mo	0	0	0	0	0	0	2+	0	0	Retarded growth; malnutrition
16	2 mo	1+	0	0	0	0	1+	1+	1+	0	0
17	2 wk	0	0	0	0	0	0	0	1+	0	0
18	3 wk	0	0	1+	0	0	2+	0	0	0	0
19	1 wk	0	2+	0	0	1+	0	1+	1+	0	0
20	1 mo	0	0	2+	0	0	0	2+	0	0	0
21	1 wk	0	1+	0	0	0	1+	1+	0	0	0
22	6 mo	0	1+	1+	0	0	0	0	0	0	Irritable
23	2 wk	1+	0	0	1+	1+	1+	1+	0	0	0
24	3.5 wk	2+	0	0	0	0	0	0	0	2+	Pale; circles under eyes
25	1 mo	1+	0	2+	0	0	0	0	1+	0	Unhappy
26	2 wk	2+	2+	3+	0	0	0	0	0	0	Recurrent otitis media
27	2 wk	0	0	0	0	0	1+	0	1+	0	Pale; weakness; anorexia; lethargy
28	2 wk	1+	0	1+	0	0	1+	1+	0	1+	Crying
29	1 wk	2+	3+	0	0	0	0	0	0	0	Perianal dermatitis
30	2 mo	1+	1+	1+	0	0	0	0	0	0	0
31	2 mo	1+	1+	0	0	0	0	1+	0	0	0
32	1 mo	0	0	0	0	0	2+	0	0	0	0
33	2-3 mo	1+	1+	1+	0	1+	1+	0	0	0	0
34	1 wk	1+	1+	1+	0	0	1+	1+	1+	0	Recurrent otitis media
35	1 wk	0	0	0	0	0	0	1+	1+	0	0
36	1.5 mo	0	4+	2+	0	0	0	0	0	0	0
37	3 wk	0	0	2+	0	0	0	0	0	0	0

* Abbreviations are as follows: Rhin., rhinitis; Asth., asthma; A.D., atopic dermatitis; Anap., anaphylaxis; Urt., urticaria; Vom., vomiting; Dia., diarrhea; Abd. Pain, abdominal pain; C.N.S., central nervous system. Figures indicate severity of symptom.

Symptoms following Milk Challenges

Age Challenged	*Rhin.*	*Asth.*	*A.D.*	*Anap.*	*Urt.*	*Vom.*	*Dia.*	*Abd. Pain*	*C.N.S.*	*Other*
11 mo	3+	0	0	0	0	1+	1+	0	0	0
10 mo	4+	4+	0	1+	4+	0	0	0	0	0
2.5 mo	0	0	0	0	0	2+	2+	1+	1+	Blood in the stool
2 mo	0	0	0	0	0	0	0	2+	0	0
2.5 mo	0	0	2+	0	0	3+	1+	0	0	0
5 mo	3+	1+	0	0	1+	0	0	0	0	0
3 wk	0	0	0	0	0	0	1+	1+	0	0
2 mo	0	0	1+	0	0	0	0	1+	0	0
1 mo	0	0	0	0	0	3+	1+	3+	0	0
10 mo	0	0	0	0	0	0	1+	0	0	0
6 yr	2+	0	0	0	0	0	0	0	0	0
6 yr	2+	0	0	0	0	1+	0	3+	0	0
4 mo	0	4+	0	0	0	0	2+	0	0	0
2.5 mo	0	0	1+	0	0	0	0	1+	0	0
14 mo	0	1+	0	0	0	2+	2+	0	0	Fever; pallor
4 mo	0	0	0	0	0	0	1+	3+	0	0
1.5 mo	0	0	0	0	0	0	1+	1+	0	0
4 mo	0	0	0	0	3+	3+	0	0	0	0
6 mo	0	0	1+	1+	4+	0	0	3+	0	0
2 mo	0	0	2+	0	0	0	2+	0	0	0
16 mo	0	1+	0	0	0	4+	2+	0	0	0
24 mo	0	0	1+	0	0	0	0	0	0	Irritable
9 mo	1+	1+	0	1+	2+	4+	1+	0	0	0
3.5 yr	2+	0	0	0	0	0	0	0	2+	Irritable, pale, circles under eyes
16 mo	1+	0	2+	0	0	1+	1+	0	0	Refused feeding irritable
16 mo	2+	3+	3+	0	0	0	0	0	0	Crying, irritable
9 mo	0	0	1+	0	0	3+	0	1+	1+	Fever, irritable, anorexia
2 mo	0	0	0	0	0	1+	0	1+	0	Crying
1.5 mo	2+	4+	0	0	0	0	0	0	0	Fever, perianal rash
6 mo	0	0	0	0	1+	1+	0	0	0	Irritable
3.5 mo	1+	1+	0	0	0	0	1+	0	0	0
1 mo	0	0	0	0	0	2+	0	0	0	4 months later developed rhinitis and asthma after fresh milk for 2 months
2 yr	1+	1+	0	1+	1+	1+	0	1+	0	0
1 mo	1+	1+	1+	0	0	1+	1+	1+	0	Irritable; Caffey's syndrome
3 mo	0	0	0	0	0	0	0	1+	0	Irritable
7 mo	0	3+	1+	0	0	0	0	0	0	Irritable
5 mo	0	0	2+	0	0	0	0	0	0	0

APPENDIX A (*Continued*)

Patient	Age of Onset	Presenting Symptoms*									
		Rhin.	*Asth.*	*A.D.*	*Anap.*	*Urt.*	*Vom.*	*Dia.*	*Abd. Pain*	*C.N.S.*	*Other*
38	2–3 mo	1+	1+	0	0	0	1+	0	0	0	0
39	1 wk	1+	0	0	0	0	0	0	1+	0	0
40	1 wk	0	1+	2+	0	0	1+	0	0	0	Failure to thrive
41	3 wk	0	0	2+	0	0	0	0	0	0	0
42	3 mo	0	3+	1+	0	0	0	0	0	0	0
43	1.5 mo	0	0	1+	0	0	0	1+	0	0	0
44	1 wk	0	0	0	0	0	1+	2+	2+	0	Sleeplessness; irritable
45	1 mo	1+	1+	0	0	0	1+	1+	1+	0	Irritable

APPENDIX B

GROUP B PATIENTS. COMPARISON OF PRESENTING ALLERGIC SYMPTOMS AND SYMPTOMS PRODUCED BY MILK CHALLENGES FOLLOWING AN ASYMPTOMATIC PERIOD

Patient	Age of Onset	Presenting Symptoms*									
		Rhin.	*Asth.*	*A.D.*	*Anap.*	*Urt.*	*Vom.*	*Dia.*	*Abd. Pain*	*C.N.S.*	*Other*
1	1 mo	1+	3+	1+	0	0	0	1+	0	0	0
2	4 mo	0	0	0	0	0	0	2+	0	0	0
3	2 mo	0	0	0	0	0	1+	0	1+	0	Screaming
4	1 mo	2+	1+	0	0	0	0	0	0	0	0
5	1 wk	0	0	2+	0	0	1+	0	0	0	0
6	1 mo	0	0	0	0	0	1+	2+	0	0	0
7	3 wk	0	2+	0	0	0	0	0	0	0	0
8	2 wk	2+	2+	0	0	0	0	2+	0	0	0
9	3 wk	0	0	1+	0	0	0	1+	0	0	0
10	2 da	1+	0	0	0	0	1+	1+	1+	0	0
11	11 yr	1+	0	0	0	0	0	0	1+	1+	Malaise, weakness
12	2 wk	1+	0	1+	0	0	0	0	0	0	0
13	3 wk	1+	0	0	0	0	0	1+	0	0	Bloody stools, anemia
14	2 wk	1+	1+	0	0	0	0	1+	0	1+	Recurrent fever, chronic colds
15	9 mo	0	0	0	1+	3+	0	0	0	0	Stridor
16	1 wk	0	0	2+	0	0	0	0	2+	0	0
17	4 mo	2+	2+	4+	0	0	0	0	0	0	0
18	4 mo	0	3+	1+	0	0	0	0	0	0	Recurrent otitis media
19	2.5 mo	1+	0	2+	0	0	0	0	0	0	0
20	3 mo	0	0	0	0	0	0	1+	1+	0	0
21	1 mo	2+	2+	4+	0	0	0	0	0	0	0
22	14 mo	1+	0	0	0	0	0	1+	0	0	Distended abdomen
23	3 mo	0	0	2+	0	0	0	0	0	0	0
24	1 mo	0	0	2+	0	0	0	0	0	0	0
25	12 yr	1+	0	0	0	0	0	0	1+	3+	Headache, fatigue hallucinations

* See footnote for Appendix A.

Symptoms following Milk Challenges

Age Challenged	Rhin.	Asth.	A.D.	Anap.	Urt.	Vom.	Dia.	Abd. Pain	C.N.S.	Other
12 mo	0	1+	0	0	0	0	0	0	0	Sleepless, crying, unhappy
1 mo	1+	0	0	0	0	0	1+	1+	0	0
17 mo	0	2+	0	0	0	0	0	0	0	0
2 yr	0	0	2+	0	0	0	0	0	0	0
5 mo	0	3+	1+	0	0	0	0	0	0	0
2 mo	0	0	1+	0	0	0	1+	0	0	0
1 mo	0	0	0	0	0	0	2+	2+	0	Crying
18 mo	1+	0	0	0	0	4+	1+	0	0	Fever, irritable

Symptoms following Milk Challenges

Age Challenged	Rhin.	Asth.	A.D.	Anap.	Urt.	Vom.	Dia.	Abd. Pain	C.N.S.	Other
2 yr	1+	0	0	0	0	0	1+	0	0	0
3.5 yr	0	0	0	0	0	0	2+	0	0	0
4 mo	0	0	0	0	0	1+	0	1+	0	Screaming
2.5 mo	0	0	0	0	0	2+	0	0	0	0
10 mo	0	0	2+	0	0	1+	0	0	0	0
10 mo	0	0	0	0	0	1+	2+	0	0	0
19 mo	0	2+	0	0	0	0	0	0	0	Fever
5 mo	0	0	0	1+	0	3+	2+	0	0	0
1.5 mo	0	0	1+	0	0	0	1+	0	0	0
5 mo	0	0	0	0	0	1+	0	1+	0	Excessive crying
13 yr	1+	0	0	0	0	0	0	0	1+	Fatigue and malaise
15 mo	0	0	1+	0	0	0	0	0	0	0
18 mo	1+	0	0	0	0	0	1+	0	0	Bloody stools
12 mo	1+	1+	0	0	0	0	1+	0	1+	Pallor
9 mo	0	0	0	1+	4+	0	0	0	0	Stridor
8.5 mo	0	0	2+	0	0	0	0	2+	0	0
8 mo	2+	2+	2+	0	0	0	0	0	1+	0
3 yr	0	3+	0	0	0	0	0	0	0	Pallor
2 yr	1+	0	2+	0	0	0	0	0	1+	0
12 mo	0	0	0	0	0	0	1+	0	0	0
12 mo	2+	3+	4+	0	2+	1+	0	0	0	0
3.75 yr	1+	0	0	0	0	0	1+	0	0	Distended abdomen
20 mo	0	0	2+	0	0	0	0	0	0	0
2 mo	0	0	2+	0	0	0	0	0	0	0
12 yr	1+	0	0	0	0	0	0	1+	2+	Headache, fatigue, irritable, listless

APPENDIX B (*Continued*)

Patient	Age of Onset	Presenting Symptoms*									
		Rhin.	Asth.	A.D.	Anap.	Urt.	Vom.	Dia.	Abd. Pain	C.N.S.	Other
26	1 mo	0	0	0	0	1+	0	0	0	1+	Edema of forehead and feet; urticaria of scalp
27	3 wk	0	0	0	0	0	1+	2+	1+	0	Bloody stools
28	2 yr	0	0	0	0	0	0	0	1+	1+	Fatigue, pallor, headaches
29	2 mo	1+	2+	0	0	0	0	0	0	0	Recurrent otitis media
30	1.5 mo	0	0	2+	0	0	1+	1+	0	1+	0
31	1 wk	1+	1+	0	0	0	1+	1+	0	0	Excessive sweating
32	1 mo	0	0	0	0	0	1+	0	0	0	Rash on buttocks
33	1 mo	1+	1+	1+	0	1+	0	0	0	0	Constant colds
34	1 mo	1+	0	0	0	0	0	0	0	0	0
35	4 mo	1+	0	1+	0	0	0	0	0	0	0
36	1 mo	0	0	3+	0	0	0	0	0	0	0
37	9 mo	0	0	2+	0	0	0	0	0	0	0
38	5 mo	0	0	1+	0	0	0	0	0	0	0
39	5 yr	3+	0	0	0	0	0	0	0	0	Listless, nervous recurrent otitis media
40	7 yr	0	0	0	0	0	1+	0	1+	0	Nausea, dizziness, leg and back pains
41	3 mo	1+	0	2+	0	0	0	0	1+	1+	0
42	3 wk	0	0	0	1+	0	0	3+	0	4+	Seizures, facial paralysis
43	1 mo	3+	1+	3+	0	0	0	0	0	1+	0
44	2 wk	1+	0	0	0	0	0	3+	0	0	Bloody diarrhea

(For Appendix C, please turn to page 440)

(Text continued from page 433)

method are serious disadvantages. However, until testing methods are developed which are more convenient and as valid as the challenge method, the diagnosis of milk allergy should be established by the combination of improvement following milk elimination plus the recurrence of symptoms following oral challenge with milk.

Subsequent reports[20,21] will deal with milk protein antibody determinations and skin testing with these specific milk proteins in this same group of children who were proven to be allergic to milk by the oral challenge method.

SUMMARY

The diagnosis of milk allergy was confirmed in 89 children by oral challenge with milk and/or purified milk proteins. Most of these patients were diagnosed as allergic during the first 2 years of life. The symptoms of milk allergy usually began during the first few months of life. Eighteen of the total patients experienced spontaneous remission of milk hypersensitivity. Multiple symptoms were produced by oral milk challenge in 77% of the patients. Vomiting, diarrhea, abdominal pain, asthma, rhinitis, and atopic dermatitis were frequently presenting and challenge symptoms. Several patients had central nervous system symptoms, urticaria, or anaphylactic reactions

Age Challenged	Symptoms following Milk Challenges									
	Rhin.	*Asth.*	*A.D.*	*Anap.*	*Urt.*	*Vom.*	*Dia.*	*Abd. Pain*	*C.N.S.*	*Other*
12 mo	0	0	0	0	1+	0	0	0	1+	Edema of the forehead
6 mo	0	0	0	0	0	1+	1+	1+	0	0
5 yr	0	0	0	0	0	0	0	1+	1+	Listless, sleepless
4.5 yr	1+	1+	0	0	0	0	0	0	0	Lethargy
4 mo	0	0	2+	0	0	0	1+	0	1+	0
10 mo	0	1+	0	0	0	0	0	0	0	0
6 mo	0	0	0	0	0	1+	1+	0	0	0
3 mo	1+	1+	1+	0	0	0	0	0	0	Refuses milk
3 mo	1+	0	0	0	0	0	0	0	0	0
4 mo	1+	0	1+	0	0	0	0	0	0	0
11 mo	2+	0	2+	0	0	0	0	0	0	0
10 mo	0	0	2+	0	0	0	0	0	0	0
7 mo	0	0	1+	0	0	0	0	0	0	0
11 yr	1+	0	0	0	0	0	0	0	1+	Listless
10 yr	0	0	0	0	0	1+	0	1+	1+	Pallor, irritable, leg pains
8 mo	1+	0	2+	0	2+	0	0	0	1+	0
2 mo	0	0	0	1+	0	0	3+	0	4+	Seizures, facial paralysis
20 mo	0	2+	2+	1+	0	4+	1+	0	0	0
4 mo	0	0	0	0	0	3+	0	0	0	Gagging, refused feeding

following milk challenge. Seventy-eight per cent of the presenting symptoms were reproduced by oral milk challenges. Challenge feedings produced symptoms not found in the history in 18% of the patients. No consistent patterns of symptom combinations were found.

The onset time of oral challenge reactions was usually within the first 12 to 24 hours. Some reactions occurred within a few minutes while others required 2 days or more before they were discernible. The duration of challenge reactions was usually between 12 to 24 hours. Allergic symptoms appeared much sooner after oral challenge in the patients who required less milk to elicit an allergic reaction.

Forty-five patients were challenged with amounts of the purified milk proteins, casein, alpha-lactalbumin, beta-lactoglobulin, and bovine serum albumin, present in the challenge doses of 100 ml skim milk. Every patient had an allergic reaction to one or more milk proteins. The frequencies of reactions were casein, 57%; BSA, 51%; beta-lactoglobulin, 66%; and alpha-lactalbumin, 54%. The reactions from oral challenge to skim milk or purified milk protein in a given patient were very similar. No correlation was found between the symptoms produced and the specific protein which produced these reactions.

By oral challenge tests few patients (13%) were allergic solely to the heat labile protein, BSA. Only two of these patients could be actually managed on a processed milk in which BSA was inactive by laboratory tests.

APPENDIX C

GROUP A PATIENTS:* RESULTS OF ORAL CHALLANGE OF EACH PATIENT WITH PURIFIED MILK PROTEINS

Patient	Protein Reactions†			
	Casein	*Alpha-lactal-bumin*	*Beta-lacto-globulin*	*Bovine Serum Albumin*
1	+	0	0	+
2	+	+	+	0
3‡	+	0	0	0
4	0	+	+	+
5	+	+	+	+
6	0	0	0	+
7	+	+	+	+
8	+	0	0	0
9	+	nd	+	+
10	+	nd	nd	0
11	+	nd	nd	nd
12	0	nd	nd	+
13	0	+	0	0
14	0	+	0	0
15	+	0	0	0
16	0	0	+	0
17	+	+	+	0
18	0	+	+	+
19	0	+	+	+
20	+	+	+	+
21	0	nd	nd	+
22	+	nd	nd	+
23	+	nd	nd	0
24	0	0	+	0
25	+	0	+	+
26	+	+	0	0
27	+	nd	+	0
28	+	+	0	+
29	+	+	0	0
30	0	nd	nd	+
31	0	0	+	0
32	0	+	+	0
33	0	+	+	+
34	+	0	+	0
35	0	0	+	+
36	+	+	+	+
37	+	+	+	+
38	+	0	0	0
39	0	nd	+	+
40	+	0	0	0
41	+	0	0	0
42	0	0	0	+
43	+	+	+	+
44	+	0	+	0
45	0	nd	nd	+

* All Group A patients had reacted previously to oral challenge with 100 ml skim milk.

† Individual protein challenge doses corresponded to he amount of that protein in 100 ml skim milk.

‡ Patient 3 was tested with the amounts of protein in 200 ml of skim milk, since she reacted to 200 but not to 100 ml of skim milk.

APPENDIX D

Case Reports*

Case 1

This case illustrates an early age of onset of milk allergy, improvement with milk elimination, multiple gastrointestinal symptoms, and the reproduction of these symptoms by oral challenge with 200 ml of milk or 6 gm of casein.

M. E. A. (Patient 3, Group A) was well until age 10 days when watery, bloody stools, perianal redness, and colic began. These symptoms continued until age 3 months when evaporated milk was discontinued and the diet limited to a soy milk. Symptoms recurred after oral challenge with each of the following foods: evaporated milk, beef, carrots, and oatmeal. The maternal grandmother, father, and older brother all had allergic asthma. An older sister had symptoms similar to those of the patient following milk ingestion for the first 2½ months of life. During the first 4 years of life, an older brother had allergic rhinitis and asthma following ingestion of corn or milk.

Physical examination, hemogram, urinalysis, and chest x-ray were normal.

Following oral challenges with 100-ml doses of skim milk, questionable reactions characterized by spitting and crying developed. No reactions were produced with separate 100-ml feedings of each milk protein. Within 20 minutes, three separate oral challenges with 200-ml skim milk and one with 200 ml (6 gm) casein produced definite reactions characterized by vomiting. By 6 hours, there was excessive crying, hyperactive bowel sounds, bloody diarrheal stools, and perianal erythema. These symptoms persisted for 12 to 24 hours. The infant continued to be irritable during the day following the test. Oral challenges with double doses of other milk proteins were negative. A repeat 100-ml skim milk challenge was negative, but a subsequent 200-ml skim milk challenge was again positive.

Case 2

This case illustrates an early age of onset of milk allergy, improvement with milk elimination, the reproduction of symptoms (rhinitis, asthma, dermatitis) by oral challenge with milk, casein and alpha-lactalbumin.

M. E. J. (Patient 26, Group A), a 14-month-old Negro female, had a persistent nasal discharge, frequent upper respiratory infections, and recur-

* These cases were studied by A.S.G.

rent wheezing since a few weeks of age. At age 4 months, a pruritic, papular, facial eruption appeared and spread to the thorax, abdomen and extremities.

Physical examination revealed an active, well-developed, well-nourished Negro female. A chronic, lichenfied weeping dermatitis was present on the face, anterior chest, and extensor surfaces of the elbows. The nasal mucosa was pale and edematous. Expiratory sibilant rales were heard bilaterally.

A milk elimination diet was begun at the age of 14 months. The symptoms promptly cleared. Twenty-five days later, the first skimmed milk challenge was done. Seven minutes following ingestion of 50-ml skim milk, profuse, serous nasal discharge and nasal mucosal edema were evident. By 10 minutes she was coughing and subcrepitant and crepitant rales were present bilaterally. By 25 minutes bilateral expiratory sibilant rales were audible. Three hours following the onset of the reaction, pruritic erythematous papules were present generally, and the patient was crying and very irritable. Severe crying lasted for several hours. The next day excoriated papules were present on the face and subcrepitant rales were distinguishable posteriorly in both lung bases. Three separate skim milk feedings and separate oral challenges with casein and alpha-lactalbumin were positive. Oral challenges with beta-lactoglobulin and BSA were negative.

Case 3

This case illustrates an early age of onset of milk allergy, improvement with milk elimination, reproduction of symptoms (asthma) following oral challenge with milk and each of the four milk proteins, the occurrence of many additional food allergies and subsequent remission of milk allergy.

O. R. (Patient 36, Group A), a 4-month-old Latin American female, was well until 6 weeks, when wheezing and a red, scaly facial rash were noted. At 7 weeks she was hospitalized for severe wheezing and respiratory distress. A chest x-ray revealed flattened diaphragms and increased radiolucency of the lungs. She improved with antibiotic and bronchodilator therapy. At 8½ weeks of age, she was readmitted with similar symptoms in critical condition. With digitalization, oxygen, and parenteral antibiotic and adrenaline therapy, she gradually improved but did not become asymptomatic. The diet consisted of evaporated milk, karo syrup, and rice cereal.

At age 4 months, when the diet was limited to soybean milk she became asymptomatic. Three months later the challenge studies were done. The first study was typical of all eight challenges with milk or milk proteins. Before each challenge, the infant was asymptomatic and the physical examination was negative. Ten minutes following ingestion of 100-ml skim milk, expiratory sibilant rales were heard in the right base posteriorly and flushing of the upper back was seen. Twenty minutes following the beginning of the test, wheezing became more generalized, but was not audible without the stethoscope. The reactions ceased within 30 minutes. Each oral challenge with casein, alpha-lactalbumin, beta-lactoglobulin, and BSA in amounts equivalent to 100 ml of milk was positive.

In turn, the patient became allergic to goat's milk, corn, pork, lamb, chicken, turkey, rabbit and goat meat. At age 11 months, these allergies, including milk, disappeared. Since then, it has been found that egg ingestion will cause wheezing.

Case 4

This case illustrates an onset of milk allergy in infancy, improvement of symptoms following milk elimination, reproduction of the early stages of atopic dermatitis by oral challenges and spontaneous remission of milk allergy.

B. D. H. (Patient 22, Group A), a 23-month-old white female was well until age 6 months when she developed dry skin, a papular pruritic rash on the face, knees and elbows, frequent coughing and irritability. Two episodes of wheezing occurred at about 1½ years. An older sibling had asthma due to corn allergy. Before milk elimination a hemogram and chest x-ray were normal. At age 23 months all symptoms disappeared following milk elimination.

At age 24 months, the first oral challenge was done. Seven minutes following ingestion of 100-ml skim milk, erythema of the popliteal areas was noted. By 15 minutes she was irritable and a papular eruption was beginning in the popliteal, gluteal, and posterolateral thigh regions. Pruritus began; the reaction increased. By 50 minutes the signs and symptoms were regressing. Similar positive reactions occurred with four separate 100-ml skim milk feedings and with 100-ml casein and BSA challenges. Three days following the BSA challenge, before the alpha-lactalbumin or beta-lactoglobulin challenges were attempted, a repeat skim milk challenge was negative. Thereafter, a full milk diet was tolerated.

Case 5

This case illustrates anaphylaxis produced by the first feeding of cow's milk at age 3 months and by subsequent feedings of trace amounts of milk, casein, beta-lactoglobulin, and alpha-lactalbumin.

M. S. A. (Patient 2, Group A), a 10-month-old white female, was the product of a full-term, uncomplicated pregnancy. She was breast fed until age 3 months. At that time, the first feeding of homogenized milk immediately caused chok-

ing, coughing, flushing and itching. She was given a soybean milk diet, which was tolerated without symptoms. Several attempts to feed very small amounts of cow's milk caused the same violent reaction. Following each episode, a pruritic papular rash developed on the face and extremities. During the last reaction at age 9 months she became pale and very weak. Because of signs of vascular collapse, the family physician immediately gave 0.1 ml of aqueous adrenaline 1:1000 and 15 mg cortisol intravenously. She improved rapidly within the next hour. A hemogram and chest x-ray at age 9 months were normal.

All subsequent oral milk challenges were done with great caution. The first oral milk challenge was done at age 10 months using 1 ml of $1:10^6$ dilution of the skim milk. Physical examination at this time was negative except for residual lichenification of the cheeks, antecubital and popliteal areas. Within 20 seconds, coughing, choking, and severe crying were evident. By 3 minutes, generalized flushing and intense pruritus began. By 5 minutes profuse nasal and oral mucoid secretions were present. At this point 0.1 ml of adrenaline, 1:1000 was given subcutaneously. The reaction subsided over the next 30 minutes. By the next day, a papular, erythematous pruritic eruption was present on the face and antecubital areas. Oral challenges of similar dilution were done with each of the four purified milk proteins. These same symptoms occurred to less than a microgram of casein, beta-lactoglobulin and alpha-lactalbumin. Three oral challenges with 20 to 40 mg of BSA were negative.

At age 2½ years, the child is still hypersensitive to minute amounts of milk feedings and shows similar reactions to egg albumen.

Case 6

This case illustrates generalized seizures and diarrhea produced by feedings of cow's milk.

A. V. (Patient 42, Group B), a 2-month-old Negro male, was the product of a full-term pregnancy complicated by preeclampsia. He was breast fed and did well until age 3 weeks when 4 ounces of homogenized milk were offered. Three hours afterward, twitching and jerking of the arms and legs were noted for 30 minutes. Ten hours later, loose, watery stools were passed. No further symptoms developed. The mother decided to continue breast feeding alone. The second milk feeding trial was at age 2 months. Immediately following this 4-oz. feeding of boiled homogenized milk, foul, watery, greenish stools began. Approximately 18 diarrheal stools were passed during the next 24 hours. Generalized seizures began 8 hours after the milk ingestion.

Physical examination revealed a convulsing male infant with a total left facial paralysis. The hydration and the remainder of the physical examination were normal. The hemogram, urinalysis, serum electrolytes, blood urea nitrogen, fasting blood sugar and cerebrospinal fluid examinations were normal. Subdural aspirations were negative. Stool, blood, and cerebrospinal fluid cultures were negative.

These tonic-clonic seizures disappeared in 24 hours, and the left facial weakness slowly improved so that by the sixth day no focal neurologic findings remained. Skull roentgenograms and an electroencephalogram 2 weeks later were normal.

Feedings were limited to soy milk. He remained asymptomatic until age 3 months, when a small amount of homogenized milk was fed inadvertently to the patient by a visiting relative. Within a few minutes seizures began. Within a few minutes, the mother gave the child a subcutaneous injection of adrenaline (0.1 ml of 1:1000). In 10 minutes the patient was seen by the physician. Generalized tonic-clonic seizures were observed. About 10 ml of milk and gastric fluid were aspirated from the stomach. In 15 minutes, 0.1 ml of adrenaline 1:1000 was given. By one hour the seizures ceased, and no abnormal neurologic findings were apparent. Since then, the milk elimination diet has been continued and he has remained asymptomatic. At age 5 months his developmental status and physical findings were normal.

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Necrosis, Cirrhosis and Cancer of Liver in Rats Fed a Diet Containing Dimethylaminoazobenzene.*

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It was recently reported[1] that dietary liver injury (necrosis, cirrhosis) seen in rats fed a synthetic ration is determined in great part by the absence of the lipotropic activity of casein. These

6 Höber, R., and Moore, E., *J. Gen. Physiol.*, 1939, **23**, 191.

* The contents of this paper have been presented by one of us (P.G.) as part of two lectures, one given on Feb. 12, 1941, before the Society of the Sigma Xi, Western Reserve Chapter, and the other on March 4, 1941, before the Duke Medical Society, Durham, North Carolina.

† S.M.A. Corporation Fellow in Biochemistry assigned to Pediatrics.

1 György, P., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 492.

changes can be prevented to a large extent by the addition of casein to the diet, but this is accomplished more effectively by the combined oral administration of cystine and choline.

During the last 2 years similar investigations have been carried out with the purpose of elucidating the cause of cirrhosis and cancer of the liver in rats brought about by the addition of butter yellow (dimethylaminoazobenzene, dissolved in oil) to a diet consisting of rice and carrots, as first described by Kinosita.[2]

The question arose whether or not the same mechanism that proved effective in the prevention of simple dietary liver injury[1] may also play a rôle in the prevention of changes in the liver which follow the administration of butter yellow.‡

Results. 1. In a control group of rats fed basal diet A, consisting of rice, carrots, and dimethylaminoazobenzene in oil (0.6 g per kilo of diet), supplemented with 20 μg daily each of thiamine, riboflavin and pyridoxine and with 100 μg daily of pantothenic acid, cirrhosis, atypical, nodular proliferation of bile ducts and carcinoma§ of the liver were a regular feature. In different groups the incidence of these changes fluctuated from 80 to 100%. However, only 40% of rats fed the same diet with the addition of 18% casein‖ showed similar changes. These observations are in close agreement with the results obtained by Kensler and his collaborators[4] just published.

2. Sixty rats were put on basal diet A (rice, carrots, dimethylaminoazobenzene) with the usual supplements of thiamine, riboflavin, pyridoxine and pantothenic acid. In addition sub-group 1 (16 rats) of this experiment received from 10 to 20 mg of choline

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2 Kinosita, R., *Trans. Soc. Path. Jap.*, 1937, **27**, 665.

‡ The special series of experiments dealing with the histologic character of these liver changes and with the effect of various vitamin-like substances including biotin, para-aminoazobenzoic acid and dried liver[3] will be reviewed in a separate paper. In this study, as well as in the continuation, as yet incomplete, of the experiments reported here, the staff of the research laboratories of the S.M.A. Corporation has taken and is continuing to take an extensive part.

3 Nakahara, W., Fujiwara, T., and Mori, K., *Gann.*, 1939, **33**, 57.

§ The lesion spoken of as carcinoma in these experiments is microscopically identical with that reported by other investigators who have used butter yellow but, so far, in our experience, it has not shown metastases and has not proved transplantable.

‖ Thiamine, riboflavin, pyridoxine and pantothenic acid have been kindly furnished by Merck & Co., Rahway, New Jersey, and casein by the S.M.A. Corporation, Chagrin Falls, Ohio.

4 Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P., *Science*, 1941, **93**, 308.

daily, sub-group 2 (12 rats) received from 25 to 50 mg of cystine daily and sub-group 3 (12 rats) received daily from 25 to 50 mg of cystine plus from 10 to 20 mg of choline. Sub-group 4 (20 rats) received no supplement. The results are summarized in Table I.

TABLE I.

Basal diet A		Supplement	Liver changes produced		
			Cirrhosis	Atypical nodular proliferation of bile ducts	Carcinoma
rice carrots thiamine riboflavin and pyridoxine pantothenic acid	dimethylamino-azobenzene (0.6 g per kg diet)	none	+++	+++	++
		cystine	+++	+++	+++
		choline	+++	++	++
		cystine and choline	+	+	0

Incidence of lesions: + slight, ++ moderate, +++ great.

3. Twenty rats kept on diet B, composed of casein 18%, cane sugar 68, melted butter fat 8, salt mixture 4, and cod liver oil 2, and supplemented again with thiamine, riboflavin, pyridoxine (20μg of each daily), with pantothenic acid (100 μg daily) and with the same amount of dimethylaminoazobenzene (0.6 g per kilo of diet) as in basal diet A, were conspicuously free of cancer or of atypical proliferation of bile ducts. Up to 175 days of the experimental period only 2 rats have shown mild patchy necrosis and slight cirrhosis[5] as the only sign of liver injury.

4. Eighty rats were put on diet C, composed of casein 6%, lard 23, cane sugar 15, cornstarch 50, salt mixture 4, cod liver oil 2, supplemented with dimethylaminoazobenzene in the same proportion as in diets A and B. The animals were subdivided into 4 groups. Sub-group 1 received no further supplement, sub-group 2 received 20 mg of choline daily, sub-group 3 received 50 mg of cystine daily and sub-group 4 received 50 mg of cystine plus 20 mg of choline. The 18 rats in sub-group 1 and the 30 rats in sub-group 2 all showed evidence of diffuse and severe hemorrhagic necrosis with or without cirrhosis. In sub-group 3, all 18 rats exhibited marked cirrhosis with or without additional necrosis, whereas in the 14 rats in sub-group 4 only 2 manifested severe cirrhosis, 3 slight cirrhotic or necrotic changes and the remaining 9 were free even of such changes.

In view of all these observations the conclusion appears to be

[5] György, P., and Goldblatt, H., *J. Exp. Med.*, 1939, **70**, 185.

warranted that the lipotropic activity of casein and in particular the combined oral administration of cystine plus choline afford a definite but not regular protection against pathological changes in the liver (necrosis, cirrhosis, atypical nodular proliferation of bile ducts, adenocarcinoma, malignant hepatoma) produced by a diet containing dimethylaminoazobenzene.

Further, it is noteworthy that no malignant changes were seen in rats fed rations containing dimethylaminoazobenzene but no rice (diets B and C). This may be due, apart from the effect of casein (present in diets B and C), either to a special carcinogenic property of rice or to other differences, such as content of butter fat, lard, sugar or corn starch in the rations used.

The experimental period lasted up to 175 days.

The experiments were performed on several hundred rats and are being continued.

13032 P

Centripetal Discharges in Dorsal and Ventral Roots Following Stimulation of Muscle by Ventral Root Volleys.

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When a ventral spinal root (L 7 or S 1) of the cat under light dial narcosis (0.5 ml/kilo Ciba) is stimulated, discharges arising in the periphery are conducted centripetally to both dorsal and ventral roots. Afferent activity consequent upon muscle contraction would be expected, but the discharges under consideration are such as to suggest that the mediation of muscle receptors is not involved. Centripetal discharges in ventral roots have been observed following the administration of eserine,[1] or prostigmine.[2] Apparently such discharges have not been observed previously in the non-eserinized preparation.

Dorsal Root Discharges. Record A of the accompanying figure shows the early centripetal activity (spike potentials 1 and 2) recorded from L 7, D. R. following a single shock to L 7, V. R. Similar discharges from the periphery may be recorded if S 1 roots

1 Dun, F. T., and Feng, T. P., *Chin. J. Physiol.*, 1940, **15**, 433.

2 Masland, R. L., and Wigton, R. S., *J. Neurophysiol.*, 1940, **3**, 269.

teine, *26*; antioxidants, *24*; sodium pyrophosphate in low concentrations, *23*). The synthesis can occur aerobically and anaerobically (in the presence of an excess of energy-rich phosphate bonds, e.g. adenosine-triphosphate, *14, 30*). The amount of acetylcholine synthesized depends on temperature (*14, 16*) and pH (optimum at alkaline pH, *29*). The enzyme is located intracellularly (*8*).

On the basis of the above data the following postulate is presented. Normally occurring constituents of cells and extracellular fluid (serum, spinal fluid) modify the amount of acetylcholine synthesized in the living organism. Further, there is a dynamic equilibrium between potentiator substances (organic phosphates, metabolites of carbohydrates and fats, amino acids, inorganic ions, hormones, vitamins) and inhibitor substances (unsaturated and higher fatty acids, aromatic and heterocyclic compounds, steroid substances, inorganic ions, some decomposition products of nucleoproteins and organic phosphates). During physiological activity the original dynamic equilibrium is disturbed, and new dynamic equilibria are established. Certain metabolites of muscle released during prolonged work decrease the synthesis of acetylcholine (*23, 25, 26*). The accumulation of such metabolites is important in the production of fatigue resulting from indirect stimulation and secondary to decreased acetylcholine synthesis.

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Granulocytopenia and Anemia in Rats Fed Diets of Low Casein Content[1]

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Granulocytopenia, correctable by crystalline *L. casei* factor (L.C.F., "folic acid"), has been found to occur occasionally in rats fed highly purified diets (*4*) and regularly when sulfonamides are included in such diets (*3, 4, 7*). Anemia (or impairment in erythropoiesis following hemorrhage) correctable by L.C.F. also has been found in rats fed sulfonamide-containing diets (*1, 3, 6*). Recently we have noted granulocytopenia in rats fed highly purified diets deficient in riboflavin and also among pair-fed, riboflavin-supplemented controls (*5*). Further investigation of this influence of inanition on the production of granulocytopenia has revealed limitation of casein intake to be a highly significant factor.

Weanling albino rats (Osborne and Mendel) were fed one of several purified diets differing only with respect to casein content. Diet No. 1055 contained no casein or protein and consisted principally of Crisco, salt mixture, and dextrose.[2] In the other diets casein (Labco) in varying amounts replaced equivalent weights of dextrose. Total white blood cell counts, polymorphonuclear granulocyte counts, and hematocrit determinations were made as previously described (*6*). For the purposes of this report, granulocytopenia was considered to be present when the polymorphonuclear granulocytes numbered 500 or less per cu. mm. Anemia was considered to be present when the hematocrit was less than 30 vol. per cent.

Of 89 rats fed the casein-free diet (No. 1055), 10

[1] Presented in part by one of us (A. K.) before the AAAS Vitamin Conference at Gibson Island, Maryland, July 1945.

[2] The casein-free diet No. 1055 consisted of anhydrous dextrose, 86.76 grams; Crisco, 8.0 grams; salt mixture No. 550[3], 4.0 grams; ferric citrate, 1.16 grams; and copper sulfate · $5H_2O$, 0.08 grams. Into this diet were incorporated 1 mg. of thiamine hydrochloride, 2 mg. of riboflavin, 1 mg. of pyridoxine hydrochloride, 4 mg. of calcium pantothenate, 2 mg. of niacin, 200 mg. of choline chloride, 0.001 mg. of biotin, and 0.4 mg. of 2-methyl-1,4-naphthoquinone. Twice weekly each rat received a supplement of 0.25 cc. of corn oil containing 2,000 units of vitamin A and 200 units of vitamin D (Natola) and once weekly 3 mg. of α-tocopherol in 0.03 cc. of ethyl laurate.

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K.) before the AAAS Maryland, July 1945. sisted of anhydrous ms; salt mixture No. ams; and copper sul- were incorporated 1 riboflavin, 1 mg. of cium pantothenate, e, 0.001 mg. of biotin, ione. Twice weekly cc. of corn oil con- 0 units of vitamin D ocopherol in 0.03 cc.

died within 19 days after starting the experimental diet. One or more blood counts were made between the nineteenth and twenty-eighth days on the surviving 79 rats. Granulocytopenia was noted in 75 rats and anemia in 68. The 4 rats without granulocytopenia and the 11 rats without anemia failed to survive 28 days on the experimental diet. The average body weight was 42.5 grams at the start of the experiment and 30.2 grams after 20 days on the diet. Of 6 and 7 rats fed diets containing 2 and 4 per cent casein, respectively, all developed granulocytopenia within 30 days. Anemia was noted only among the rats fed the 2-per cent casein-containing diet. Seven of 8 rats fed an 8-per cent casein-containing diet developed granulocytopenia within 45 days; none had anemia.

TABLE 1

GRANULOCYTOPENIA AND ANEMIA IN RATS FED PROTEIN-FREE DIETS AND PREVENTION WITH CASEIN

Group*	Diet	No. of rats	No. of rats with granulocytopenia‡	No. of rats with anemia‡
A	0 per cent casein	8	8	6§
B	0 per cent casein — 20 γ L.C.F. daily†	8	8	7§
C	18 per cent casein — pair-fed with Group A ..	8	2	0
D	18 per cent casein	8	0	0

* The 4 groups were equal with respect to sex, litter, and weight distribution. Food intake was ad libitum in groups A, B, and D.
† This crystalline fermentation product (*2*) was administered by pipette to each rat from the outset of the experiment.
‡ Noted within 28 days.
§ The rats which failed to develop anemia died after 19 to 25 days.

Data on the influence of restriction of food intake and the effect of L.C.F. (*2*)[3] administered preventively are in Table 1. These data indicate no significant preventive action by L.C.F. Severe restriction of an 18-per cent casein-containing diet (Group C) failed to produce blood dyscrasias in 6 of 8 rats. The 2 cases of granulocytopenia noted in this group may have resulted from inadequate casein intake.

Several materials were tested for their effectiveness in correction of granulocytopenia developed in rats fed the casein-free diet No. 1055. Treatment was administered daily for 4 days. A recount was made on the day following the last treatment. For the purposes of this report, a response was considered "positive" when the granulocytes numbered 1,000 or more cells per cu. mm. Further details and results of treatment are in Table 2. Rats which f vive the treatment period are not consid no treatment was administered, there wa sive decline in granulocyte count and hem terminating in death. Of 13 rats treate talline L.C.F. or a liver concentrate of

TABLE 2

TREATMENT OF GRANULOCYTOPENI

Treatment	No. of rats	No. of rats with positive response
L.C.F.*—100 γ	8	2
L.C.F.†—200 γ subcutaneously	2	0
L.C.F.†—100 γ + L.C.F. conc.‡ —50 γ	3	0
Casein diets—18 per cent or 30 per cent	9	0
L.C.F.†—100 γ + L.C.F. conc.‡ —50 γ + casein diet—18 per cent	3	3
L.C.F.*—100 γ + casein diet—18 per cent	5	5
L.C.F.§—100 γ + amino acid mixture	6	6

* Crystalline fermentation L.C.F. (*2*) or (Stokstad). No differences were observed be ity of the two substances in these experiment
† Crystalline fermentation L.C.F. (*2*).
‡ Contained in 0.058 grams of liver concer
§ Synthetic L.C.F. (Stokstad).

2 reached granulocyte levels of 1,000 cel Granulocyte values declined in all of 9 containing casein at levels of 18 or 3(place of the casein-free diet No. 1055. administration of L.C.F. combined wit an 18-per cent casein-containing diet r nificant increases in granulocyte count rats. Similarly treatments with L.C.F. the dietary administration of a mixtu amino acids[5] produced significant incre locyte count in each of 6 rats. Data ment of anemia are incomplete and the presented at this time.

Further study is required to determi of the amino acids responsible for th poietic activity found in casein or in purified amino acids. Such data ma elucidation of the mechanism of acti factor and the amino acids in the form locytes.

[3] The crystalline fermentation L.C.F. (*2*), synthetic L.C.F., and L.C.F. concentrate from liver used in these studies were furnished through the courtesy of Drs. E. L. R. Stokstad and B. L. Hutchings, of Lederle Laboratories, Inc.

[4] Five and 4 rats were fed diets contain and 30 per cent casein, respectively. Aver of these diets per rat was 3.6 grams.

[5] To mixture XII-c of W. C. Rose and S. S. *Chem.*, 1942, 143, 115) containing 18 amino 7.8 grams of dl threonine and 7.8 grams of Of this final mixture, 26.7 grams (representi "active" amino acids) replaced an equal we in the casein-free diet No. 1055. Average this diet per rat was 2.7 grams.

Summary

Severe granulocytopenia and anemia were developed uniformly in rats fed protein-free diets. Casein (18 per cent) prevented these dyscrasias, but crystalline *L. casei* factor ("folic acid") did not prevent them. In the correction of granulocytopenia in rats fed protein-free diets, *L. casei* factor alone was only slightly effective, diets of higher casein content (18 or 30 per cent) were ineffective under the experimental conditions described. However, *L. casei* factor combined with an 18-per cent casein-containing diet or *L. casei* factor combined with a mixture of purified amino acids were found to be highly effective in correcting the granulocytopenia.

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The Presence and Significance of a Leukopenic Factor in Inflammatory Exudates

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A number of inflammatory conditions are accompanied by a fall in the number of circulating white blood cells, a so-called state of leukopenia. Fitz-Hugh and Krumbhaar (*1*) regard agranulocytosis as the result of an arrested development of leukocytic elements. The disease involves lymphoid elements as well as granulocytes. These authors therefore speak of the condition as a pernicious leukopenia. A profound leukopenia referable to a virus infection has been recently described to occur in cats (*2, 3*). It is interesting to note on close scrutiny the frequent occurrence of some infection accompanying an agranulocytic process.

The writer has demonstrated the presence of an injury factor located in, or at least closely associated with, the euglobulin fraction of inflammatory exudates (*5*). This substance has been termed necrosin. Recent studies indicate its more frequent recovery in exudates from a severe area of inflammation in which there is usually an appreciable degree of acidity (*7*). The whole euglobulin fraction of exudates not only induces marked cutaneous injury, but likewise it causes in dogs a marked degree of fever and a profound leukopenia (*5, 6*). Subsequent investigations have revealed that the pyrogenic property of the whole euglobulin fraction of exudates is really not referable to necrosin, but that this fever-inducing capacity is caused by a completely different, but closely associated substance, termed by the writer pyrexin (*6*). The present preliminary communication indicates that in inflammatory exudates there exists a leukopenic factor which is not one of the biological attributes of necrosin *per se*. It is closely associated with pyrexin. Yet, it can readily be dissociated, at least to a large extent, from this pyrogenic factor. The presence of such a leukopenic factor in inflammatory exudates may in large part explain, perhaps, the state of leukopenia accompanying numerous inflammatory processes. The leukocytosis-promoting factor present in exudates may well mask the ultimate effect of this leukopenic factor (*4*). In brief, the final blood picture accompanying an acute inflammatory process may to a large extent depend on the relative concentration of either the leukocytosis-promoting factor (LPF) or the leukopenic factor now under discussion, both of which factors are produced at the site of an acute inflammation.

An inflammatory exudate at an acid pH will, when injected into the circulation of a dog, tend to induce a rapid and sharp fall in the number of circulating leukocytes. This is a conspicuous feature within the first hour or so. The average fall in 8 experiments has been found to be 3,778 white blood cells per cubic millimeter or 32.3 per cent. Pyrexin, as isolated from such exudates, is the fraction obtained which has been found to induce a marked leukopenia. The average fall in 10 experiments is 9,980 white blood cells per cubic millimeter, a drop of 79 per cent. It is possible that the simultaneous presence of the LPF in the whole exudate counteracts somewhat the full effectiveness of the leukopenic factor. Such a state of affairs would account for the more striking effect obtained with pyrexin where the LPF is absent. Purified necrosin or normal blood serum utterly fails to induce any such drop in the leukocyte count. Within the usual period of study (about 6 hours) the maximum decrease in the number of circulating leukocytes is, under normal circumstances, negligible.

An attempt has been made to dissociate the leukopenic factor from pyrexin. Some recent evidence indicates that the latter is, or is at least associated with, a polypeptide. It is possible that the leukopenic factor also belongs to this group, especially since it is derived from pyrexin. For this reason pyrexin has been partially hydrolyzed with 0.1 N HCl for about 10 to 15 minutes in an effort to determine

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INFLUENCE OF CASEIN AND OTHER AGENTS ON THE PRODUCTION OF RENAL LESIONS IN RATS BY SULFADIAZINE AND ACETYLSULFADIAZINE [1]

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Renal lesions have been produced by sulfadiazine in experimental animals (*1–3*). In humans treated with sulfadiazine, similar lesions have been observed (*4–8*). The lesions generally have been attributed to the precipitation of insoluble drug in the tubules and lower urinary tract. Sodium bicarbonate, which increases the solubility of sulfadiazine by creating an alkaline medium (*9*), has been used clinically (*10, 11*) and experimentally (*12*). Urea has also been shown to increase the solubility of sulfonamides (*13, 14*) and to prevent renal lesions in rats fed acetylsulfapyridine (*15*). Since the completion of the present work, it has been reported (*16*) that diuresis from sodium chloride or "alkalinizing salt mixtures" was corrective in acute renal obstruction produced in rats by sulfadiazine administration.

Our interest in this problem arose from the observation, made in the course of nutritional experiments, that rats fed sulfadiazine-containing diets of low casein content (10 percent) for a 30-day period regularly developed unusually severe renal lesions. We have found that the incidence and severity of these lesions were reduced by increasing the casein content of the diet or by giving urea, sodium bicarbonate, or sodium chloride. Increased conjugation of sulfadiazine and high renal concentrations of conjugated sulfadiazine have been noted in connection with development of the lesions. This relationship between conjugated sulfadiazine and renal lesions has been studied further by the administration of acetylsulfadiazine.

METHODS

Albino rats of Wistar and Osborne and Mendel strains were weaned at about 22 days and fed one of several purified diets (table 1). In some experiments, rats were fed sulfonamide-containing diets at weaning while in other studies the rats first were fed control diet No. 836 for 1 or 3 weeks and then were fed the sulfonamide-containing diets. Food intake was always ad libitum. Water intake was either ad libitum or restricted.

[1] From the Division of Physiology and the Pathology Laboratory, National Institute of Health.

TABLE 1.—*Percentage composition of diets* [1]

Diet No. [1]	Sulfadiazine	Acetylsulfadiazine [2]	Potassium chloride	Sodium chloride	Sodium bicarbonate	Urea	Casein (Smaco)	Sucrose
803	1						10	77
819							10	78
832	1						20	67
833	1					5	10	72
835	1				4		10	73
836							20	68
843	1			4			10	73
855		1					10	77
856		1				5	10	72
857		1			4		10	73
858		1		4			10	72
859		1					30	57
866	1						30	57
884	1		4				10	73
898		0.75					10	77.25
899		.50					10	77.50
900		.25					10	77.75
906		.50				5	10	72.50
907		.50		4			10	73.50
908		.50			4		10	73.50
909		.50					30	57.50
920	2				4		10	72
933	3				4		10	71
943	4				4		10	70

[1] Each diet included Crisco 8 percent, salt mixture No. 550 (*17*) 4 percent, and vitamins in the following amounts per 100 gm. of diet: 1 mg. of thiamine hydrochloride, 2 mg. of riboflavin, 1 mg. of pyridoxine hydrochloride, 4 mg. of calcium pantothenate, 2 mg. of niacin, 200 mg. of choline chloride, and 0.4 mg. of 2-methyl-1, 4-naphthohydroquinone diacetate. Twice weekly each rat was given an oral supplement of 0.25 cc. of corn oil containing 2,000 units of vitamin A and 200 units of vitamin D (Natola), and once weekly 3 mg. of α-tocopherol in ethyl laurate.

[2] The acetylsulfadiazine used in these studies was furnished through the courtesy of Dr. E. H. Northey and Mr. W. O. Brewer, Calco Chemical Division, American Cyanamid Co.

Litter mates of the same sex and closely comparable weight were always used in setting up experimental and control groups. The average weight of weanling rats was 36 gm. and that of 6-week-old rats was 120 gm.

The experimental period was usually 30 days. At the end of this period, surviving rats were decapitated. Animals which appeared moribund during this period also were decapitated in order to obtain fresh tissues for chemical and histologic study. Tissues were fixed in 3.7-percent aqueous formaldehyde, embedded in paraffin, sectioned, and stained with eosin-azure and van Gieson's picrofuchsin. The right kidney was usually used for histologic study and the left was weighed and used for determination of sulfadiazine concentration. In the present report, the renal lesions were graded "very slight to moderate" and "severe," depending upon the extent and degree of tubular dilatation, cast formation, epithelial degeneration, and the amount of neutrophil exudation and interstitial reaction.

Sulfadiazine was determined by the methods of Marshall and co-workers (*18, 19*). Tissues were homogenized in a Waring blendor and filtrates were prepared as previously described for cecal contents (*20*). Values were expressed on the basis of wet weight. Determinations of the ratio of free to total sulfadiazine in the urine were made on small freshly voided specimens.

The term "conjugated sulfadiazine" is used in this report to denote the form of sulfadiazine which can be determined by the Bratton and Marshall method (*18*) only after hydrolysis.

RESULTS

SULFADIAZINE

Production of lesions.—Renal tubular lesions of unusual severity were observed in rats fed the 1-percent sulfadiazine, 10-percent casein-containing diet (No. 803) for the 30-day experimental period. In a group of 122 weanling and 4-week-old rats fed diet No. 803, 114 had severe lesions and 8 had milder ones. This group includes 20 rats which formed part of a study on the prevention of renal lesions (table 2). The incidence of lesions was not as high among older, heavier rats. Of ten 6-week-old rats fed diet No. 803, 5 had severe lesions, 1 had a moderate lesion, and 4 had none (table 3).[2] The incidence of lesions appeared to be the same whether the casein used was crude or purified.

TABLE 2.—*Production and prevention of renal lesions in weanling rats fed 1-percent sulfadiazine-containing diets for 30 days*

	Water intake, ad libitum						Water intake restricted ("paired")		
	Diet No. 819	Diet No. 803	Diet No. 833	Diet No. 835	Diet No. 843	Diet No. 832	Diet No. 803	Diet No. 833	Diet No. 832
	Control	10-percent casein	Urea	Sodium bicarbonate	Sodium chloride	20-percent casein	10-percent casein	Urea	20-percent casein
Number of rats	10	[1]10	[1]5	5	[1]5	[1]10	[1]10	[1]10	5
Weight gain in 30 days (gm. per rat)	51	12	25	27	23	34	11	16	24
Food intake (gm./day)	6.9	3.6	4.9	4.2	3.7	3.4	3.7	3.6	4.1
Water intake (cc./day)	5.2	8.8	14.0	7.9	13.2	13.1	9.4	9.1	9.2
Blood sulfadiazine concentration (mgm. percent): 15 days (free)	0	65	48			56	64	45	47
30 days (free)	0	44	38	22	32	41	47	29	34
(conjugated)	0	4	0	1	0	2	3	0	0
Urinary pH: 10 days	6.6	6.6	6.5	7.7	6.5	6.3	6.3	6.3	6.4
20 days	6.7	6.6	6.3	7.8	6.8	6.3	6.4	6.6	6.4
30 days	7.0	6.7	6.6	8.1	6.6	6.2	6.7	6.4	6.3
Kidney sulfadiazine concentration (mgm. percent): (free)	0	167	60	5	56	134	188	89	95
(conjugated)	0	134	33	24	181	255	380	0	51
Kidney weight (mgm.)	355	597	419	291	385	637	542	329	427
Kidney lesions: Absent	0	0	2	5	2	1	0	8	1
Very slight to moderate	0	2	2	0	1	3	2	1	3
Severe	0	7	0	0	1	5	7	0	1

[1] 1 rat in this group failed to survive the 30-day experimental period.

The figures given here are average values.

Only a brief account of the pathological character of the renal lesions will be given here. More detailed observations, including the histogenesis of the lesions in these rats, are reported elsewhere (*21*).

The damaged kidney was usually enlarged. The average kidney weight of a group of 10 rats fed the 1-percent sulfadiazine, 10-percent casein-containing diet (No. 803) was 597 mgm. (range: 450–815 mgm.), while that of a group of

[2] Thirteen rats varying from 9 to 16 weeks of age and 97 to 300 gm. in weight were fed diet No. 803 for the experimental period of 30 days after having received control diet No. 836 from weaning. Severe lesions were noted in 8 rats, mild lesions in 3 rats, and 2 rats were normal.

litter mates fed the control, 10-percent casein-containing diet (No. 819) was 355 mgm. (range: 265–415 mgm.) (table 2). Upon inspection, the kidney surface appeared smooth but was completely covered with white specks less than 1 mm. in diameter. The intervening tissue appeared pale tan in color. In sagittal sections, white chalky streaks were noted in a radial pattern extending from papilla to cortex.

Histologically, the involvement was generally limited to collecting tubules, distal convoluted tubules, and the ascending limb of Henle's loop. The tubules were greatly dilated and contained casts and amorphous debris. In special preparations sulfadiazine and acetylsulfadiazine crystals were demonstrated in the dilated tubules. Their epithelium showed flattening, degeneration, or proliferation, and the surrounding interstitium often showed leucocyte infiltration and fibroblast proliferation. Glomerular and vascular lesions were not noted and deposition of calcium was rare.

Calculi and hydronephrosis were never observed grossly in association with these lesions. However, single or multiple bladder calculi were found in 11 rats in these studies in which these renal lesions did not occur.

Lesions noted in other tissues in these rats have been reported (*22*).

Pyuria was noted in all rats which developed lesions. Freshly voided, uncentrifuged urine usually contained 100 to 200 white blood cells per high power field (300 diameters). Red blood cells and casts were noted infrequently. Repeated microscopic examination of the urinary sediment in each of the 10 control rats (table 2) failed to reveal any abnormal elements.

Prevention of lesions.—Increase of the casein content of the diet from 10 to 20 or 30 percent, or inclusion of urea, sodium bicarbonate, or sodium chloride[3] in the diet were noted to have preventive actions on the development of lesions (tables 2 and 3). Lesions were completely prevented in 6-week-old rats by each of these substances while in weanling rats only sodium bicarbonate was completely effective. In considering how these substances might act in preventing lesions, several factors have been studied. Some of these factors are food and drug intake, water intake and urine output, solubility of sulfadiazine and acetylsulfadiazine, and absorption of sulfadiazine.

Food intake was measured and from this the sulfadiazine intake was calculated. It was found generally to be less in the rats which developed lesions than in the rats in which the lesions were prevented (tables 2 and 3).

Water intake was either ad libitum or paired. When the intake was ad libitum (table 2), each of the preventive agents except sodium bicarbonate resulted in an augmented water intake. However, the water intake of individual rats within a group showed no correlation with the development of lesions. In another experiment, the water intakes of litter mates fed the urea-containing diet (No. 833) and the 20 percent casein-containing diet (No. 832) were restricted to the

[3] Potassium chloride was also found to have a preventive action. No lesions were observed in four 6-week-old rats fed the potassium chloride-containing diet (No. 884) for the 30-day experimental period. The average values were 771 mg. for kidney weight, 48 mg. percent (free) and 18 mg. percent (conjugated) of sulfadiazine in the kidney, and 28 mg. percent (free) and 0 mg. percent (conjugated) of sulfadiazine in the blood.

intakes of the litter mates fed the basic experimental diet (No. 803) (table 2). As an added check, measurements of urine outputs were made and no significant differences between the various groups were found. It may be noted that the preventive actions of urea and casein were no less than and perhaps even superior to the preventive actions observed in the previous experiment when water intake was unrestricted and, therefore, greater. Thus the preventive actions could not be attributed to diuresis.

TABLE 3.—*Production and prevention of renal lesions in 6-week-old rats fed 1-percent sulfadiazine-containing diets for 30 days*

		Diet No. 803, 10-percent casein		Diet No. 833	Diet No. 835	Diet No. 843	Diet No. 866
		Lesions present	Lesions absent	Urea	Sodium bicarbonate	Sodium chloride	30-percent casein
Number of rats		6	4	10	10	10	10
Weight gain in 30 days (gm. per rat)		−20	+4	+2	+1	+7	+13
Food intake (gm./day)		5.1	7.7	7.6	7.2	7.6	7.3
Water intake (cc./day)		14.1	13.4	12.2	13.5	13.7	13.7
Blood sulfadiazine concentration (mgm. percent).	5 days (free)	64	74	43	44	32	44
	10 days (free)	60	70	34	45	39	46
	20 days (free)	40	50	28	26	19	35
	20 days (conjugated)	0	0	1	0	0	0
	30 days (free)	52	50	33	21	23	25
	30 days (conjugated)	0	0	0	0	0	0
Conjugated sulfadiazine fraction in urine (percent).	3 days	55	27	34	26	33	33
	6 days	52	20	28	27	28	33
	9 days	47	23	28	26	36	27
	12 days	51	31	23	27	32	28
	18 days	64	30	28	25	28	34
	24 days	52	27	26	18	29	27
	29 days	51	23	23	12	19	25
Kidney sulfadiazine concentration (mgm. percent).	(free)	76	110	37	23	19	68
	(conjugated)	201	5	1	0	4	2
Kidney weight (mgm.)		989	586	667	601	593	658
Kidney lesions	absent	0	4	10	10	10	10
	very slight to moderate	1	0	0	0	0	0
	severe	5	0	0	0	0	0

These 10 groups of 5 litter mates were prepared for 3 weeks after weaning on a 20-percent casein sulfadiazine-free diet (No. 836).
Water intake was "paired" for each group of litter mates during the 30-day experimental period.
The figures given here are average values.

The increased solubility of sulfadiazine and acetylsulfadiazine in alkaline media is well known (*9*). The average pH values of the urines of rats fed the sodium bicarbonate-containing diet (No. 835) were from 7.7 to 8.1, while those of rats on the basic experimental diet (No. 803) were from 6.6 to 6.7 (table 2). The preventive agents other than sodium bicarbonate did not elevate the urinary pH values above those of rats fed diet No. 803. However, it was found that sodium chloride depressed the in vitro solubilities of sulfadiazine and acetylsulfadiazine, while urea, as has been shown (*14*), increased them. The "salting out" effect of sodium chloride has been reported for other compounds such as benzoic acid (*23*, *24*), phenol, and phenyl acetic acid (*24*). The preventive actions of all of these agents, therefore, cannot be accounted for entirely on the basis of an increased solubility.

In order to obtain a rough indication as to whether any of the preventive agents might interfere with absorption of sulfadiazine, determinations were made of the sulfadiazine concentration in cecal contents. Pooled samples of cecal contents from all rats in table 3 were used for this purpose. Sulfadiazine concentrations for the groups of rats fed the various diets expressed as grams sulfadiazine (free) per 100 gm. of wet cecal contents were as follows: 2.44 for the basic, experimental diet (No. 803), 0.95 for the urea-containing diet (No. 833), 0.56 for the sodium bicarbonate-containing diet (No. 835), 0.75 for the sodium chloride-containing diet (No. 843), and 0.96 for the 30 percent casein-containing diet (No. 866). From these data, there is no indication that these various agents depress the absorption of sulfadiazine. It has also been found possible to prevent in rats fed a 20-percent casein-containing diet (No. 836) the severe renal lesions which result from the subcutaneous administration of sulfadiazine (1 mgm. per gm. body weight per day) to rats fed a control, 10-percent casein-containing diet (No. 819).

TABLE 4.—*Preventive action of sodium bicarbonate in rats fed 2-, 3-, and 4-percent sulfadiazine-containing diets for 30 days*

	Diet No. 803	Diet No. 920	Diet No. 933	Diet No. 943
	Sulfadiazine, 1 percent; sodium bicarbonate, 0 percent	Sulfadiazine, 2 percent; sodium bicarbonate, 4 percent	Sulfadiazine, 3 percent; sodium bicarbonate, 4 percent	Sulfadiazine, 4 percent; sodium bicarbonate, 4 percent
Number of rats	18	5	5	5
Weight gain (gm. per rat per day)	−0.23	+0.54	+0.63	+0.49
Blood sulfadiazine concentration (mgm. percent). 15 days (free)	64	38	39	55
Blood sulfadiazine concentration (mgm. percent). 30 days (free)	66	35	43	63
Blood sulfadiazine concentration (mgm. percent). 30 days (conjugated)		0	0	
Conjugated sulfadiazine fraction in urine (percent)[2]	65	23	20	19
Kidney sulfadiazine concentration (mgm. percent). (free)	98	46	169	176
Kidney sulfadiazine concentration (mgm. percent). (conjugated)	813	3	8	17
Kidney weight (mgm.)	892	394	413	490
Kidney lesions. absent	0	5	5	2
Kidney lesions. very slight to moderate	0	0	0	[3] 3
Kidney lesions. severe	5	0	0	0

[1] 3 rats in this group died during the experimental period.
[2] Determinations were made on 2 to 4 specimens obtained from each rat during the last 4 days of the experimental period.
[3] These 3 lesions were all slight (±).

These rats were prepared for 1 week after weaning on a 20-percent casein, sulfadiazine-free diet (No. 836) and then placed on this experiment.
The figures given here are average values for all rats which survived the 30-day experimental period.

Effect of preventive agents on blood sulfadiazine levels.—Rats fed diets containing urea, sodium bicarbonate, sodium chloride, or higher levels of casein had much lower blood sulfadiazine levels than rats fed the basic, experimental diet (No. 803) (tables 2 and 3). This effect was noted when water intake was either ad libitum or restricted. The data in table 3 show that rats fed sodium bicarbonate, sodium chloride,

or extra casein in their diets had average blood-sulfadiazine concentrations of 21 to 25 mg. percent at 30 days, that rats fed the urea-containing diet (No. 833) had a concentration of 33 mg. percent, while rats given no protective agent (diet No. 803) had a concentration of 51 mg. percent. However, it is noteworthy that in this latter group the blood levels of rats which did not develop lesions were as high as the levels of rats that did develop lesions.

TABLE 5.—*Further studies on the preventive action of sodium bicarbonate in rats fed a 4-percent sulfadiazine-containing diet*

	Diet No. 803 Sulfadiazine 1 percent			Diet No. 943 Sulfadiazine 4 percent Sodium bicarbonate 4 percent		
Rat number	1	2	3	4	5	6
Weight gain in 30 days (gm. per rat)	+3	−9	+2	+15	+13	+15
Percent of absorbed sulfadiazine recovered in urine	94	84	93	71	81	86
Urine sulfadiazine concentration (mgm. percent). (free)	84	102	96	281	329	247
Urine sulfadiazine concentration (mgm. percent). (conjugated)	78	92	89	82	35	80
Percent of recovered sulfadiazine in conjugated form	48	47	48	23	10	25
Blood sulfadiazine concentration (mgm. percent). (free)	43	50	51	46	41	37
Blood sulfadiazine concentration (mgm. percent). (conjugated)	2	5	1	0	0	0
Liver sulfadiazine concentration (mgm. percent). (free)	28	33	30	23	22	23
Liver sulfadiazine concentration (mgm. percent). (conjugated)	0	0	0	0	0	0
Heart-lung sulfadiazine concentration (mgm. percent).[1] (free)	37	40	40	32	34	30
Heart-lung sulfadiazine concentration (mgm. percent).[1] (conjugated)	0	3	0	0	0	0
Kidney sulfadiazine concentration (mgm. percent). (free)	53	64	66	211	92	82
Kidney sulfadiazine concentration (mgm. percent). (conjugated)	139	172	246	0	0	0
Kidney weight (mgm)	810	710	950	700	520	430
Kidney lesions	Severe	Severe	Severe	Slight	Slight	None

[1] The heart and both lungs were determined as a single tissue specimen.

Rats Nos. 1, 2, and 3 were litter mates of the same sex and weight of rats Nos. 4, 5, and 6 respectively. They were prepared as in table 4. At the conclusion of the 30-day experimental period, urinary studies were made during a 5-day "balance" period. The rats were sacrificed following this 5-day period.

In order to test whether one of the preventive agents was effective in the presence of high blood sulfadiazine levels, diets were fed which contained sodium bicarbonate and higher percentages of sulfadiazine (table 4). It was not until sulfadiazine was incorporated in the diet (No. 943) at a 4-percent level that blood concentrations were reached which were comparable with concentrations obtained in feeding the diet No. 803 (containing 1 percent sulfadiazine but without sodium bicarbonate). Slight renal lesions were produced.

Conjugation of sulfadiazine as related to the development of lesions.—Increased conjugation of sulfadiazine (as indicated by the conjugated sulfadiazine fraction in the urine) was found in all rats which developed severe renal lesions, but was not found in rats which did not develop lesions. In rats with severe renal lesions the conjugated sulfadiazine fraction in the urine was in the neighborhood of 50 percent, while in rats with slight or no renal lesions it was about 25 percent (tables 3, 4, and 5). However, no relationship between the actual urinary concentration of conjugated sulfadiazine and the development of lesions was noted in studies with sodium bicarbonate (table 5).

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High concentrations of the conjugated sulfadiazine fraction in the kidney were found to be associated invariably with the development of severe lesions and were not found in the absence of lesions. This relationship was noted without exception in all the data obtained in these studies (tables 2, 3, 4, and 5). Thus, in a group of rats fed the 4-percent sulfadiazine, 4-percent sodium bicarbonate-containing diet (No. 943) in which renal lesions were very slight or absent, the average concentrations of free and conjugated sulfadiazine were 176 and 17 mg. percent, respectively (table 4). A group of rats fed the basic experimental, 1-percent sulfadiazine-containing diet (No. 803) all developed severe lesions. In the kidneys of these rats, the average concentrations of free and conjugated sulfadiazine were 98 and 813 mg. percent, respectively. It was also noted in the individual values which went to make up the average values in tables 2, 3, and 4, that in some kidneys without lesions, the total sulfadiazine concentrations (with free sulfadiazine predominant) were higher than in some kidneys with lesions (with conjugated sulfadiazine predominant).

Recovery from lesions.—Histologic studies of early and late recovery stages in rats with severe renal lesions have been made and are reported elsewhere (*21*). The rats used in these studies had developed severe renal lesions after ingestion of the basic experimental, 1-percent sulfadiazine-containing diet (No. 803) for the 30-day experimental period starting from weaning. At the conclusion of the experimental period, unilateral nephrectomy was carried out. The rats recovered on a diet (No. 819) lacking in sulfadiazine but otherwise identical to diet No. 803. In these studies the earliest histologic evidence of recovery was a disappearance of casts. The dilated tubules resumed a normal appearance or collapsed and atrophied. Tubular atrophy was most marked just beneath the capsule and produced a pitted appearance. Interstitial edema and leucocyte infiltration disappeared. Interstitial fibrosis increased. In rats killed after 3 months' recovery on diet No. 819, the kidneys showed atrophy of as many as one-half of the subcapsular tubules and interstitial fibrosis of the cortex and medulla.

Recovery studies in the continued presence of sulfadiazine were also carried out in 13 rats with severe lesions. After unilateral nephrectomy, 2 rats were continued on the same basic experimental diet (No. 803), 5 on a urea-containing diet (No. 833), and 6 on a sodium bicarbonate-containing diet (No. 835). All the rats on diets No. 803 and No. 833 died within 5 days with no evidence of repair in the remaining kidney. Of the 6 rats fed the sodium bicarbonate-containing diet (No. 835), 3 died early, while 3 survived and were sacrificed 40 days later. By this time the acute lesions had disappeared and only residual stigmata (cortical scars, tubular atrophy) remained. Renal deposits of conjugated sulfadiazine were absent, conjugation was reduced by about 80 percent and the blood concentration was reduced by about 50 percent (table 6).

Renal lesions with sulfathiazole.—Feeding of sulfathiazole-containing diets has resulted in renal lesions similar to those produced by sulfadiazine. Of 24 rats fed a 1-percent sulfathiazole, 10-percent casein-containing diet (identical to diet No. 803 except that sulfathiazole replaced sulfadiazine), 22 died in an average of 8 days (range: 3–18 days). Slight to moderate renal lesions were observed in 11 of these rats. Of 12 litter mates fed a similar sulfathiazole-containing diet with the casein level raised to 25 percent, all but 1 survived the 30-day experimental period and lesions were noted in only 2 rats. When urea (5 percent) was included in the 1-percent sulfathiazole, 10-percent casein-containing diet and fed to 4 rats, all survived the 30-day experimental period without evidence of renal lesions. When methionine (0.5 percent) was included in the 1-percent sulfathiazole, 10-percent casein-containing diet of 4 pair-fed litter mates, all died within 4 days and 2 of these had renal lesions.

TABLE 6.—*Influence of sodium bicarbonate on recovery from renal lesions*

	Diet No. 803 Before recovery period [1] sulfadiazine 1 percent			Diet No. 835 After recovery period [2] sulfadiazine 1 percent sodium bicarbonate 4 percent		
Rat number	7	8	9	7	8	9
Blood sulfadiazine concentration (mgm. percent) (free)	56	73	64	35	24	36
Conjugated sulfadiazine fraction in urine (percent)	[3] 72	[3] 44	[3] 59	[4] 9	[4] 13	[4] 13
Kidney sulfadiazine concentration (mgm. percent). (free)	32	24	42	18	9	9
Kidney sulfadiazine concentration (mgm. percent). (conjugated)	141	86	201	18	9	18
Kidney weight (mgm.)	695	510	710	405	540	630
Kidney lesions	severe	severe	severe	none	none	none

[1] These values were obtained at the conclusion of the 30 day experimental period on diet No. 803. Unilateral nephrectomy was performed at this time and diet No. 835 was fed.
[2] These values were obtained 40 days after unilateral nephrectomy.
[3] Average of 5 daily determinations from the twenty-sixth to the thirtieth experimental days.
[4] Average of 5 daily determinations from the sixty-sixth to the seventieth experimental days.

ACETYLSULFADIAZINE

Production of lesions.—Administration of acetylsulfadiazine in a 10-percent casein-containing diet (No. 855) resulted in more severe renal lesions and shorter survival than the administration of sulfadiazine in such a diet (No. 803). Diets were fed which contained acetylsulfadiazine at levels of 0.25, 0.50, 0.75, and 1.00 percent (table 7).

TABLE 7.—*Production of renal lesions in 6-week-old rats fed 0.25-, 0.50-, 0.75- and 1.00-percent acetylsulfadiazine-containing diets*

	Diet No. 900, 0.25 percent	Diet No. 899, 0.50 percent	Diet No. 898, 0.75 percent	Diet No. 855, 1.00 percent
Number of rats	6	6	6	6
Number of rats surviving the 30-day experimental period	6	6	5	2
Weight gain (gram per rat per day)	+1.3	0	−1.4	−3.2
Blood sulfadiazine concentration (mgm. percent). (free)	2	9	14	17
Blood sulfadiazine concentration (mgm. percent). (conjugated)	3	8	13	17
Muscle sulfadiazine concentration (mgm. percent). (free)		3	6	7
Muscle sulfadiazine concentration (mgm. percent). (conjugated)		4	6	6
Liver sulfadiazine concentration (mgm. percent). (free)		5	8	10
Liver sulfadiazine concentration (mgm. percent). (conjugated)		6	10	10
Cecal contents sulfadiazine concentration (mgm. percent). (free)	18	50	69	116
Cecal contents sulfadiazine concentration (mgm. percent). (conjugated)	131	469	1,088	1,849
Kidney sulfadiazine concentration (mgm. percent). (free)	0	10	19	34
Kidney sulfadiazine concentration (mgm. percent). (conjugated)	43	259	368	514
Kidney weight (mgm.)	930	1,277	1,541	1,878
Kidney lesions: absent	1	0	0	0
Kidney lesions: very slight to moderate	3	0	0	0
Kidney lesions: severe	2	6	5	2

The figures given here are average values for rats which survived the 30-day experimental period.

Of 12 rats fed the 1-percent acetylsulfadiazine-containing diet (No. 855), only 2 survived the 30-day experimental period. The lesions observed in these 2 rats and in some of those dying as early as 13 days after starting the experiment were of extreme severity (tables 7 and 8). Even at a level of 0.5 percent (diet No. 899), acetylsulfadiazine appeared to be as effective as 1-percent sulfadiazine (diet No. 803) in the production of severe renal lesions (compare data in table 3 with data in tables 7 and 9).

TABLE 8.—*Effects of preventive agents on the production of renal lesions in 6-week-old rats fed 1 percent acetylsulfadiazine-containing diets*

		Diet No. 855, 10 percent casein	Diet No. 856, urea	Diet No. 857, sodium bicarbonate	Diet No. 858, sodium chloride	Diet No. 859, 30 percent casein
Number of rats		6	6	6	6	6
Number of rats surviving the 30-day experimental period		0	1	4	0	0
Survival in days (range)		10–20	5–16	11	8–13	8–12
Kidney sulfadiazine concentration (mgm. percent).	free	19	15	11	13	17
	conjugated	707	467	418	324	308
Kidney lesions	very slight to moderate	1	5	4	4	2
	severe	5	1	2	2	4

These 6 groups of 5 litter mates were prepared for experiment as in table 3.
Water intake was "paired" for each group of litter mates.
The figures given here are average values.

Values for the concentration of acetylsulfadiazine in cecal contents (table 7) in rats fed varying amounts of the drug are of the same order of magnitude as values reported for sulfadiazine under somewhat similar conditions (*20*). This finding suggests that the greater renal toxicity of acetylsulfadiazine is not due to greater intestinal absorption.

The blood concentrations of total sulfadiazine were much lower in rats fed acetylsulfadiazine than in rats fed a similar amount of sulfadiazine. About 50 percent of the total sulfadiazine in the blood was in the free form. In liver and muscle tissues, the free form also constituted about 50 percent of the total drug concentration. Since less than 10 percent of the urinary sulfadiazine was found to be in the free form (table 9), the relatively high ratios of free to conjugated compound in the blood and other tissues are probably due to the more rapid renal clearance of the conjugated compound. It is noteworthy that, despite very low blood concentrations, severe renal lesions were developed in most rats.

Anatomically these renal lesions were similar to those seen with sulfadiazine. It was noted, however, that with acetylsulfadiazine more tubules were involved and the inflammatory changes were more marked. Leucocytes were more numerous both in the tubules and in the interstices. Tubular epithelium showed more degeneration. The most extreme renal damage of the entire study was encountered in rats given acetylsulfadiazine.

TABLE 9.—*Effects of preventive agents on the production of renal lesions in 6-week-old rats fed 0.5 percent acetylsulfadiazine-containing diets*

			Diet No. 899, 10 per cent casein	Diet No. 906 urea	Diet No. 908, sodium bicarbonate	Diet No. 907, sodium chloride	Diet No. 909, 30 per cent casein
Number of rats			6	[1] 6	6	[2] 6	6
Blood sulfadiazine concentration (mgm. percent).	10 days	(free)	4.4	2.0	0.4	1.7	1.1
		(conjugated)	4.9	2.0	1.1	2.5	2.1
	20 days	(free)	7.0	2.6	1.0	4.5	1.5
		(conjugated)	11.7	4.5	3.5	4.8	2.8
	30 days	(free)	8.9	3.2	1.3	2.3	2.1
		(conjugated)	10.5	1.4	.8	1.3	1.7
Heart-lung sulfadiazine concentration [3] (mgm. percent).		(free)	5.6	1.7	1.0	.1.3	2.0
		(conjugated)	7.0	1.1	.6	.8	1.0
Conjugated sulfadiazine fraction in urine [4] (percent)			93–99	97–100	95–99	97–99	97–99
Kidney sulfadiazine concentration (mgm. percent).		(free)	10	6	0	2	0
		(conjugated)	277	362	101	106	117
Kidney weight (mgm.)			1,096	891	666	820	737
Kidney lesions	absent		0	0	0	0	1
	very slight to moderate		0	1	6	2	3
	severe		6	3	0	1	2

These 6 groups of 5 litter mates were prepared for experiment as in table 3.
Water intake was "paired" for each group of litter mates.
The figures given here are average values for all rats which survived the 30-day experimental period.

[1] Two rats in this group died during the experimental period.
[2] Three rats in this group died during the experimental period.
[3] The heart and both lungs were determined as a single tissue specimen.
[4] These figures represent the range of averages of values obtained 5, 10, 15, 20, and 25 days after start of the experiment.

Prevention of lesions.—Casein, urea, sodium bicarbonate, and sodium chloride, each of which were found to have some effectiveness in prevention of the renal lesions caused by sulfadiazine were tested in similar dietary concentrations for their effectiveness in prevention of lesions caused by acetylsulfadiazine.

One-percent acetylsulfadiazine-containing diets were fed to 5 groups of 6-week-old litter mate rats (6 per group) (table 8). The basic, 10-percent casein-containing diet (No. 855) was fed to one group. A urea-containing diet (No. 856) was fed to a second group, a sodium bicarbonate-containing diet (No. 857) to a third group, a sodium chloride-containing diet (No. 858) to a fourth group, and a 30-percent casein-containing diet (No. 859) to the remaining group. Poor appetite and weakness were noted early. Only 5 of 30 rats survived the 30-day experimental period. Four of the five rats were in the sodium bicarbonate-fed group and one was in the urea-fed group. Renal lesions were noted in all rats and no significant influence could be attributed to any of the 4 agents studied. Early death with the resultant lack of time for the full development of lesions made comparisons difficult.

One-percent acetylsulfadiazine-containing diets were fed to five groups of weanling rats (five per group). The experimental conditions were the same as for 6-week-old rats. The toxicity of the drug for weanlings was greater than for the 6-week-old rats. None of the rats survived the 30-day experimental period. Twenty-one of the twenty-five rats died within 15 days. The remaining four rats, all of which were in the sodium bicarbonate-fed group, died after 26 to 29 days. As with the 6-week-old rats, renal lesions were noted in all the animals.

Five-tenths percent acetylsulfadiazine-containing diets were fed to five groups of 6-week-old rats (6 per group) (table 9). Here too, the conditions, except for the acetylsulfadiazine content of the diets, were the same as in the experiments with 1-percent acetylsulfadiazine-containing diets. Most rats survived the 30-day experimental period. As in a previous experiment (table 7), severe renal lesions were noted in all rats fed the basal, 10-percent casein-containing diet (No. 899). Sodium bicarbonate was effective in preventing the development of severe renal lesions but ineffective in preventing the development of milder ones. The 30-percent casein-containing diet (No. 909) appeared to have some degree of protective action. Only two of six rats had severe lesions, three had milder ones, and one rat had no lesions. In the group fed urea and sodium chloride, some of the rats did not survive the experimental period and the data are inadequate for determining any preventive effect.

The conjugated sulfadiazine fraction in the urine determined at 5-day intervals during the 30-day experimental period was from 93 to 97 percent of the total drug (table 9). Since the amount of deacetylation (as judged by the excretion of free sulfadiazine) was so small, the present data are not adequate for determining whether the various agents had any significant influence on deacetylation.

The effects of the various agents in depressing the sulfadiazine blood concentration are clearly shown (table 9) both with the free form as in earlier experiments (table 3) and with the conjugated form. Values for drug concentrations in the heart and lungs were lower but showed the same pattern as the blood values.

DISCUSSION

Reduction of the casein content of a sulfadiazine-containing diet to a level of 10 percent has permitted the uniform production of severe renal tubular lesions. These lesions are similar in general character to those reported in experimental animals (*1–3*) and observed in man (*4–8*) following the administration of sulfadiazine. Sodium bicarbonate, sodium chloride and urea, in addition to casein, have been found to exert a preventive action on the development of these renal lesions. The preventive effect of these agents is another illustration of the need for consideration of dietary factors in the interpretation of pharmacologic data (*25–27*).

High concentrations of sulfadiazine in the blood have been noted along with the development of lesions. These high levels have been greatly reduced by the agents which exert preventive actions on the development of lesions. However, it appears likely that even if the magnitude of the blood concentration exerts an important influence on the development of renal lesions, there are other significant factors as well. In some rats with high blood levels lesions have not developed (table 3), and lesions have been prevented by sodium bicarbonate when the administration of large doses of sulfadiazine resulted in high blood values (table 4). In addition, extremely severe lesions were developed by the administration of acetylsulfadiazine even though relatively low blood concentrations were achieved (table 9).

The reduction of blood levels by sodium bicarbonate and sodium chloride may be explained in part by an increase in renal clearance as shown for sulfamerazine in dogs (*28, 29*). Clinical studies (*30*) have also indicated some depression of blood level and increased urinary excretion of sulfadiazine after the oral administration of sodium bicarbonate. Reduction of blood concentration of sulfanilamide by increasing the casein content of the diet has been reported in rats (*25, 26*) and mice (*31*). It appears suggestive that this casein effect may be accounted for to some extent by the action of urea which results from its catabolism.

The influence of the preventive agents on the therapeutic effectiveness of sulfadiazine as a result of the decrease in sulfadiazine concentration has not been determined in these studies. It is of interest to note that Rosenthal has found an impairment in the therapeutic effectiveness of sulfanilamide when the casein content of the diet was increased (*31*).

Increased conjugation of sulfadiazine and high renal concentrations of conjugated sulfadiazine have been noted whenever a severe lesion has developed, but were not found in the absence of a lesion. Although isolation and identification of the conjugated sulfadiazine fraction has not been made in these studies, it is probable that it consists largely of acetylsulfadiazine. The administration of acetylsulfadiazine under the same conditions as sulfadiazine has indicated this conjugated compound to be far more toxic both in regard to the production of renal lesions and the survival of the animals. This greater toxicity of acetylsulfadiazine is in accord with data reported in studies on rats (*2*) and with data contained in clinical reports (*4, 6, 11*). It is noteworthy that, unlike other acetylated sulfonamides, acetylsulfadiazine is more soluble than the free compound.

The same agents (sodium bicarbonate, sodium chloride, urea, and casein) which reduced the sulfadiazine blood levels also decreased the degree of conjugation and the renal concentration of conjugated sulfadiazine. Although there may be an important causal relationship between blood level and conjugation, it appears that other factors may also be operative.

It seems clear that the final development of renal lesions may be governed by a number of factors. The results of the present studies have suggested important influences by blood level and conjugation. While the value of increasing the solubility of the drug in the tubules has been confirmed, it has also been shown that sodium chloride,

despite its "salting out" effect on both sulfadiazine and acetylsulfadiazine is effective in the prevention of renal lesions. It appears likely too that other significant factors, not considered here, are operative in the production of renal lesions by sulfadiazine.

SUMMARY AND CONCLUSIONS

Sulfadiazine (1 percent) in a purified diet of low casein content (10 percent) fed to rats for 30 days has resulted in the uniform production of severe renal lesions.

Casein, urea, sodium bicarbonate, and sodium chloride have been found to exert preventive actions on the development of these renal lesions, despite restriction of water intake. Sodium bicarbonate was found to be the most effective of these agents under the specific conditions of this study.

The blood sulfadiazine concentration has been reduced by each of the preventive agents mentioned. The magnitude of the blood sulfadiazine concentration may influence the production of these renal lesions, but there appear to be other significant factors as well.

In experiments with sodium bicarbonate, severe renal lesions have been prevented, even when high blood sulfadiazine concentrations resulted from the feeding of a 4-percent sulfadiazine-containing diet.

Increased conjugation of sulfadiazine and high renal concentration of conjugated sulfadiazine have been noted to be associated invariably with these severe lesions and have never been noted when lesions were prevented.

Acetylsulfadiazine, despite its greater solubility and lower blood concentration than free sulfadiazine, was found to be far more toxic than free sulfadiazine as judged by the incidence and severity of renal lesions and by survival.

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Protein Overload Nephropathy

In Rats Subjected to Unilateral Nephrectomy

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Response of partially nephrectomized rats to the feeding of different concentrations and types of protein has been evaluated. In uninephrectomized Sprague-Dawley rats, 20% supplements of crude casein, soybean meal, or peanut meal in a ground commercial diet contributed to an increase in the incidence of glomerular degeneration, tubular dilatation, and hyaline cast formation when fed for periods of 150 days or more. Feeding for periods beyond 150 days did not seem to exaggerate the glomerular lesions. Since severe obliteration of glomeruli can be produced without leukocytic infiltration or proliferation of endothelial cells, this type of protein nephropathy is best characterized as an example of nutritional glomerulosclerosis.

IT HAS been shown that aging is capable of inducing degeneration in glomeruli and tubules of rats.[1] In longevity studies, ad libitum ingestion of nutritionally adequate diets promoted the development of nephritis[2] or glomerulonephrosis.[3] The incidence and severity of such renal degeneration was reduced by food restriction.[2,3] In shorter feeding experiments, both the quality and quantity of dietary protein was capable of modifying the function and morphology of the kidney.[4-7] Apparently, the stimulus for renal hypertrophy which resides in casein is not due solely to increased urinary excretion of nitrogen.[6] Even renal failure has been produced in weanling rats following the ingestion of a mixture of gelatin, casein, and corn gluten.[8]

Unilateral nephrectomy also fosters a gradual loss of renal parenchyma and contributes to the development of glomerulosclerosis.[9,10] The induction of degeneration in glomeruli and tubules can be accelerated by combining the feeding of excess protein with uninephrectomy in rats.[11] While experimental evidence suggests that excess protein may be nephrotoxic, it has not been resolved how many nephrons have to be removed, what concentration of protein must be fed, or the minimal time required for the development of cortical degeneration. To explore further these parameters, it was

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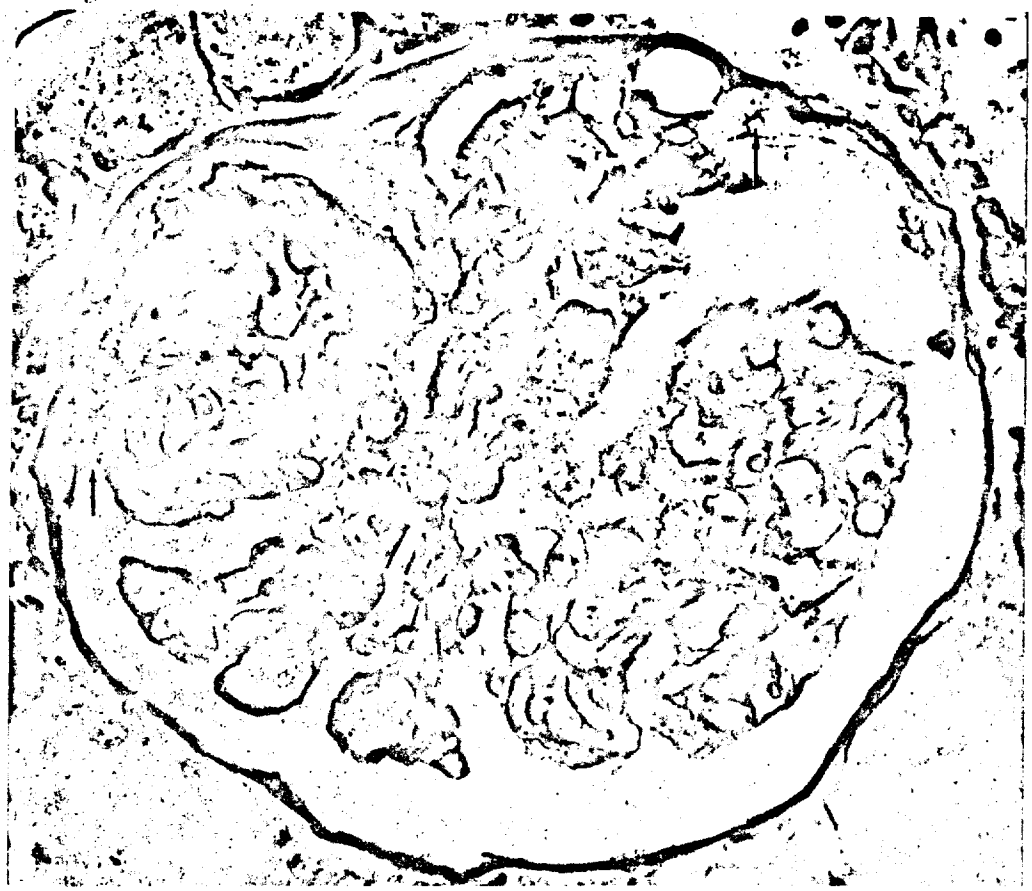

Fig 1.—Glomerulus showing delicate adhesions (arrows), only faintly PAS-positive, between two epithelia. Mesangial (M) regions of affected lobules appear expanded. Remainder of mesangial and basement membrane structures are relatively free from alteration (PAS, × 600).

decided to subject young rats to ablation of renal tissue and the ingestion of different types of protein for variable intervals of time.

Methods

Male Sprague-Dawley rats weighing 100 to 150 gm were subjected to extirpation of renal tissue while under pentobarbital sodium anesthesia (25 mg/kg body weight). The left kidney was removed in all rats. In addition, in some rats, a cotton ligature was placed around the inferior pole of the right kidney and the parenchyma distal to the ligature was excised. After surgery, one group of rats was fed a ground commercial diet with a 25% protein concentration. (Lab-Blox, Allied Mills Inc.) Others received ground diet supplemented with 200 gm crude casein, peanut meal, or soybean protein. Soybean protein supplement was fed also to six rats not subjected to extirpation of renal tissue. On this regimen, practically all of the rats grew satisfactorily and appeared healthy until some of them developed bronchopneumonia. At autopsy, the weight of the animals and the kidney were recorded. The kidneys were cut traversely through the hilum for microscopic inspection. All of the thoracic and peritoneal organs were examined for gross alterations and samples of abnormal tissue were kept for microscopic examination. The tissues were fixed in phosphate-buffered formaldehyde solution, trimmed, and impregnated with paraffin. Sections were cut at 4μ and stained with hematoxylin-eosin. All kidney sections were stained with the PAS technique. In order to more fully appreciate differences in cortical degeneration, glomeruli were counted to indicate how many had undergone some degree of sclerosis. While the extent of sclerosis was not identical in individual glomeruli, nevertheless, an expression of the num-

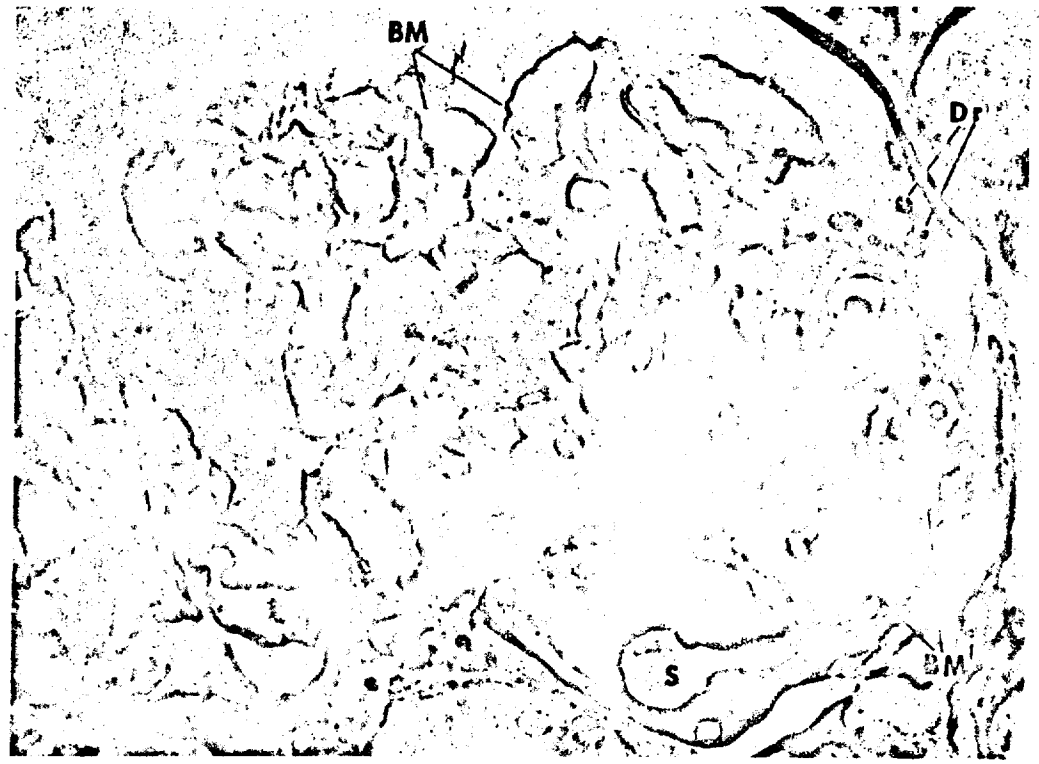

Fig 2.—Widened mesangium (M') in affected portion of glomerulus, compared with more normal mesangium (M) in left portion. Pleated convolutions of peripheral capillary basement membrane (BM') in affected lobule may also be compared with thin basement membrane (BM) elsewhere. Deep recess of Bowman's space (S) is noted. "Hyaline droplets" (Dr) are evident in cytoplasm of epithelium. Adhesions are noted in lower right portion of photomicrograph (PAS, × 850).

bers involved is helpful in making some approximation of relative cortical degeneration in the different groups of rats. In most instances, 135 glomeruli were counted and the number with sclerosis recorded. On those occasions when less or more than 135 were counted, the numbers of sclerotic glomeruli were calculated on the basis of 135. When more extensive renal cortical deterioration was encountered, 20 rats' kidneys fixed in formaldehyde solution were sectioned with a freezing microtome and stained with oil red O. Because brown pigment was frequently observed in epithelial cells of the proximal convoluted tubules in sections stained with hematoxylin-eosin, 17 representative samples were stained for iron with Perls' technique.

Photomicrography was performed using a ribbon-filament light source with a green filter, a variable-focus condenser, and a dry apochromatic objective, N.A. 0.95, on a stand. Primary photographic images were recorded at a magnification of 500 diameters; then central fields were enlarged photographically to either 600 × or 850 ×.

Results

Gross and Microscopic Observations.—Pertinent data such as growth following unilateral nephrectomy, duration of feeding, protein supplement, kidney weight at autopsy, and the microscopic findings in 25 rats selected from 52 animals are shown in Table 1.

Following nephrectomy, ground commercial diet was fed to ten rats for periods ranging from 151 to 254 days, for an average of 197 days. Five of ten rats lost appreciable weight after the fourth month due to the presence of bronchopneumonia. At autopsy, the weight of the remaining kidneys ranged from 1.6 to 2.4 gm. The number of glomeruli in ten rats varied from 2 to 38 per 135 glomeruli with an average of 15. The kidneys were considered to be normal in two animals.

Fig 3.—Glomerulus nearly completely obsolescent, with only a few persistent capillary loops in left portion of photomicrograph. Mesangial matrix material (M) is much increased, and adhesions are laced by numerous PAS-positive strands (arrows). Hyaline droplets (Dr) are again evident (PAS, × 850).

Ground commercial diet with crude casein was fed to 14 uninephrectomized rats for periods ranging from 150 to 247 days, for an average of 186 days. Although initial growth was satisfactory at autopsy, appreciable losses in weight occurred in seven rats due to bronchopneumonia. Kidney weights varied from 2.2 to 3.4 gm. Two of 14 kidneys were tan in color. Data from five representative rats are recorded in Table 1. Sclerotic glomeruli in 14 rats varied from 10 to 82 with an average of 30 per 135 glomeruli. Rats with tan kidneys on gross inspection had, on microscopic examination, more extensive glomerulosclerosis and tubular degeneration. Data from seven rats with an estimated 65% renal ablation not shown in Table 1 nor included in the statistical analyses were fed casein supplement for periods ranging from 127 to 243 days for an average of 163 days. Four rats attained a satisfactory maximum weight, whereas the others did not. At autopsy, six of seven kidneys were tan in color. Renal weights varied from 2.1 to 3.6 gm. Sclerosis counts in these seven rats varied from 21 to 120 per 135 glomeruli, with an average of 73. On the basis of microscopic observations, it is apparent that when more than one kidney is removed, the extent of glomerulosclerosis is enhanced.

Soybean supplemented diets were fed to six control rats and nine rats subjected to unilateral nephrectomy. Data from six

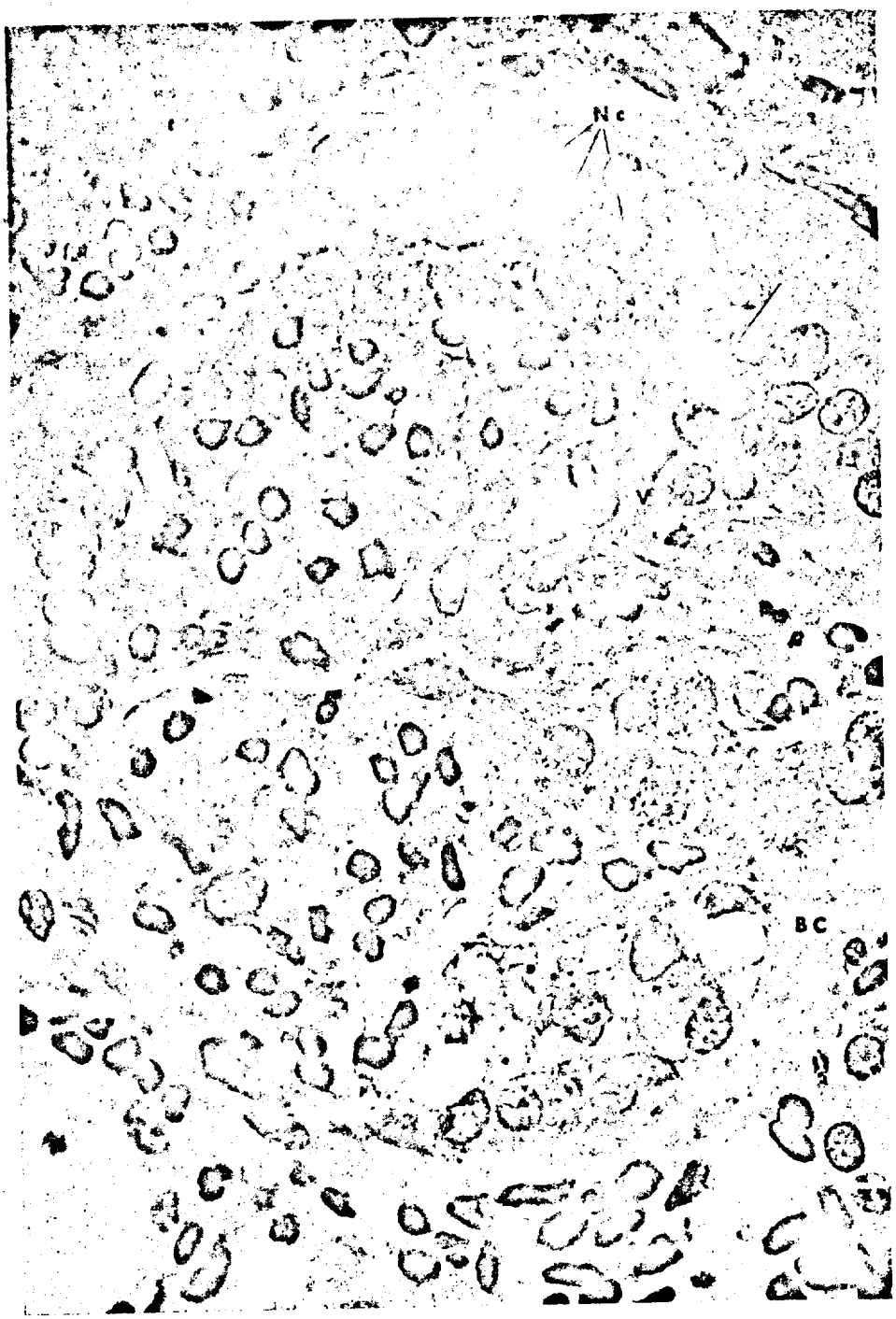

Fig 4.—Occasional glomeruli showed extreme hyperplasia of epithelium, as illustrated here, with virtual obliteration of Bowman's space. Nuclei of these cells are large and round, have sparsely distributed chromatin, and have prominent nucleoli (Nc). Mitotic figure is also seen (arrow). Both parietal (P) and visceral (V) layers appear to contribute to the process, since they can be identified by position. Endothelial and mesangial cells within glomerulus are conspicuously not participating in hyperplastic response. Bowman's capsule (BC) is thickened (hematoxylin-eosin, × 850).

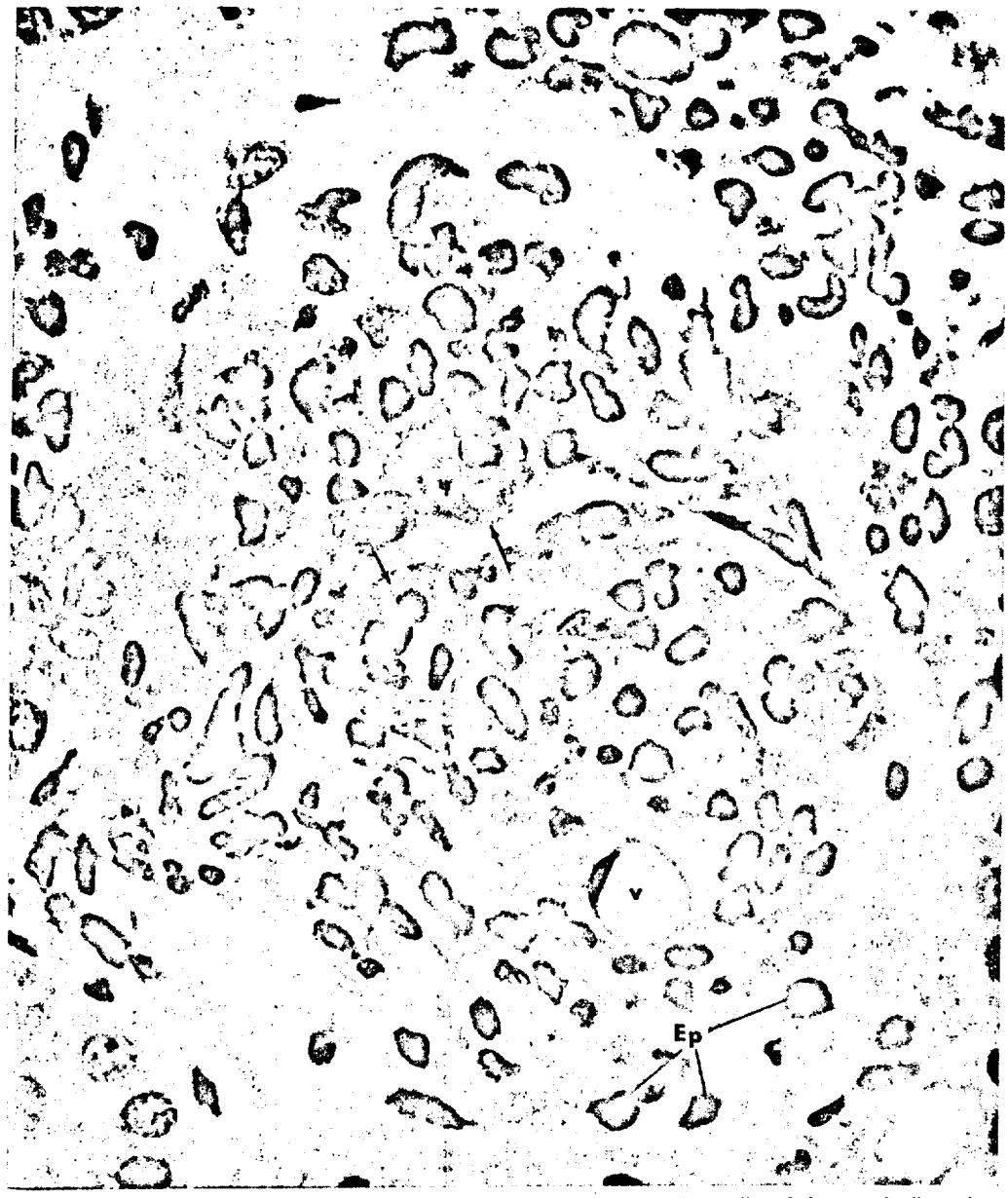

Fig 5.—This glomerulus may represent further stage of process depicted in Fig 4. Collapsed capillary loops are circumferentially adherent to Bowman's capsule, which is very thickened in some areas. Epithelial cells (Ep) are now very inconspicuous. Only a few capillary loops remain, with walls of increased dimensions (arrows). An infiltrate of lymphocytes and a few histiocytes have gathered about the capsule at upper right. Vacuolated cell (v) is unexplained (hematoxylin-eosin, × 850).

control animals fed a similar diet for 150 to 200 days, for an average of 190 days, are not shown in Table 1. Growth and autopsy weights were normal, except in one rat which developed bronchopneumonia. The combined kidney weights varied from 2.9 to 3.6 gm. Sclerosis counts varied from 0 to 11 with an average of 4 per 135 glomeruli in rats with two kidneys. Nine rats with one kidney were fed soybean supplement for 159 to 208 days, for an average of 185 days. Five rats

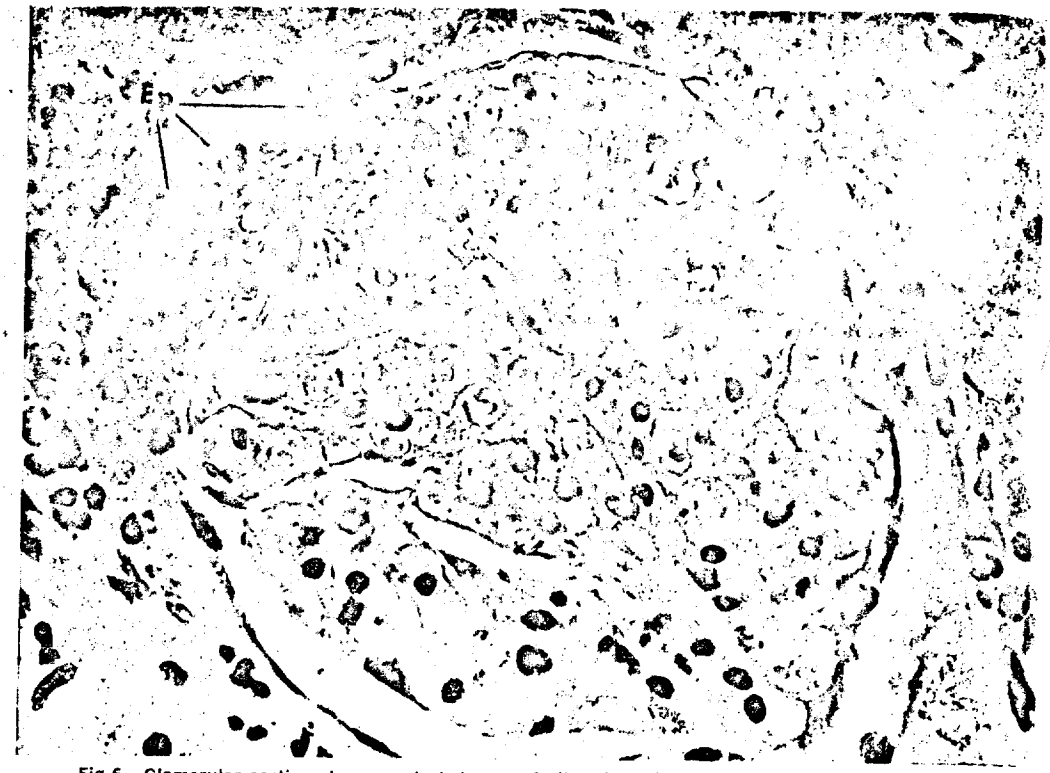

Fig 6.—Glomerular section shows modest degree of alteration: cluster of hyperplastic epithelial cells (Ep) at upper left, forming adhesion between visceral and parietal epithelium (hematoxylin-eosin, × 600).

typical of this group are shown in Table 1. Growth and autopsy weights were considered to be normal except for four rats with pneumonia. The renal weights varied from 2.8 to 3.7 gm. Renal ratios of body weight were somewhat elevated over those observed in rats fed the commercial diet. On microscopic inspection, the incidence of sclerosis varied from 13 to 79 with an average of 37 per 135 glomeruli.

Peanut meal supplement in a commercial diet was fed to six rats for 171 to 189 days, with an average of 179 days. The maximum and autopsy weights were similar to those observed in control rats. Renal weights varied from 2.3 to 3.1 gm. Sclerosis counts in six rats varied from 7 to 76 for an average of 30 per 135 glomeruli. Inspection of the kidney ratio values reveals that appreciable glomerulosclerosis can develop without marked increase in renal weight.

In kidney sections fixed in formaldehyde solution, which were cut while frozen and stained with oil red O, minimal to moderate quantities of fat were found in sclerotic glomeruli and epithelial cells of the proximal convoluted tubules. Paraffin sections of kidney with more extensive cortical degeneration which were stained with Perls' stain always contained hemosiderin in the atrophic epithelial cells of the proximal convoluted tubules.

On microscopic inspection, the glomeruli manifested one or more features of degeneration. The mesangium, although not strongly PAS-positive, was often expanded and occasionally adherent to hyaline and fibrillar aggregates at the parietal epithelium (Fig 1 to 3). Bowman's capsule was frequently thickened and sclerotic (Fig 4 and 5). Commonly, sclerosis of the capsule was associated with an eosinophilic precipitate in Bowman's space and extensive degeneration

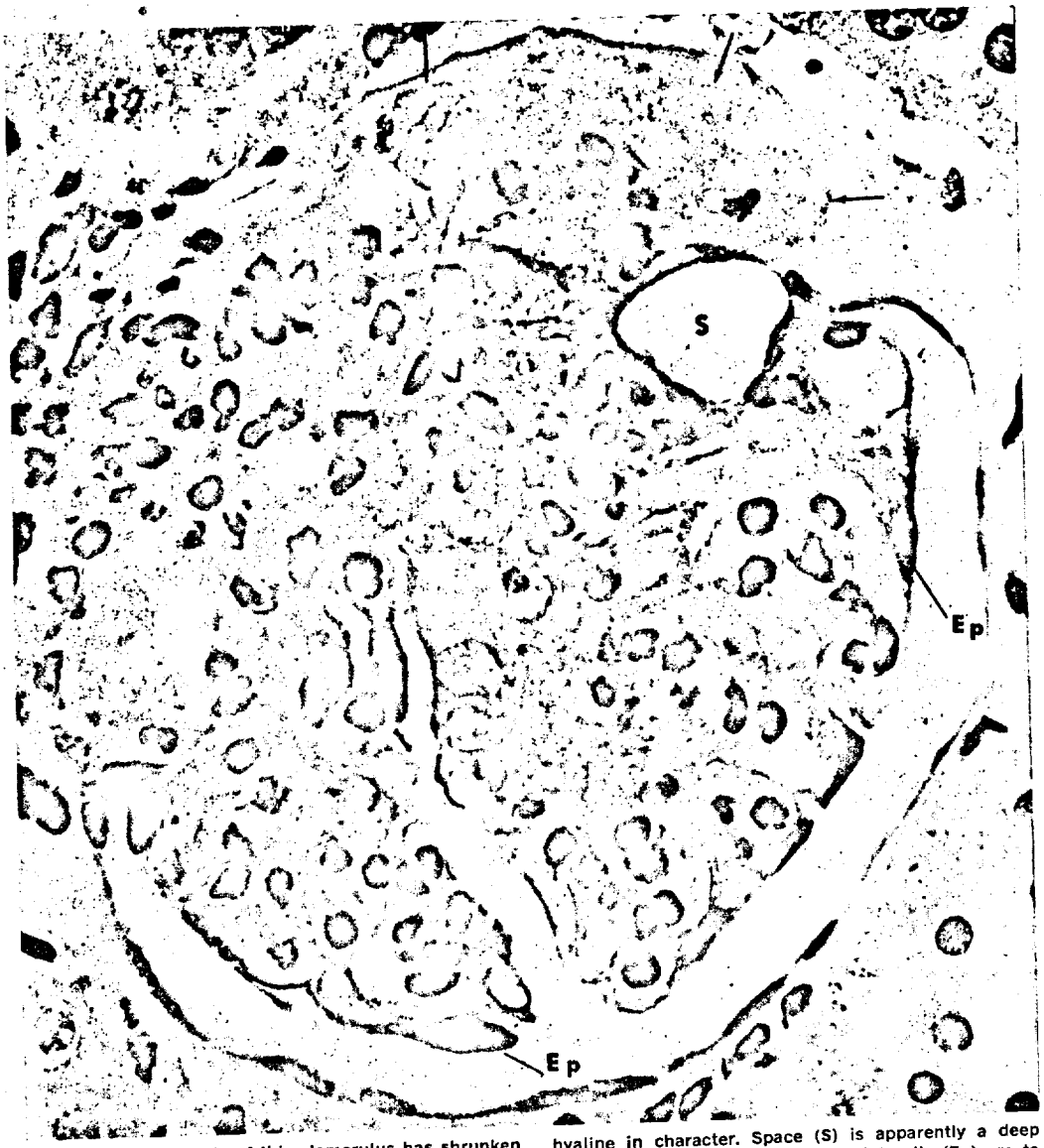

Fig 7.—Upper lobule of this glomerulus has shrunken and become sparse with cells except for several residual mesangial (?) nuclei. Periphery of former capillary loops is marked by arrows. Their capillary lumens are filled with very fine filamentous substance, nearly hyaline in character. Space (S) is apparently a deep recess of Bowman's space. Epithelial cells (Ep) are to be compared with those in Fig 4 (hematoxylin-eosin, × 850).

of glomerular epithelial cells. Occasionally, proliferation of parietal cells of Bowman's capsule acquired the appearance of microcrescents (Fig 4 and 6). Many glomerular capillary lumens were obliterated by precipitation of eosinophilic and fibrillar material, accompanied by apparent fusion of hyperplastic parietal and visceral epithelium (Fig 7). Frequently, PAS-positive aggregates tended to accumulate in the visceral epithelial cells (Fig 2 and 3) and in the proximal convoluted tubules. The tendency of epithelium to become hyperplastic was not limited to that of the glomerulus but was found in the convoluted tubules, both proximal and distal, where abundant cell multiplication resulted in polyp-like pro-

Table 1.—Response of Rats With Unilateral Nephrectomy to the Feeding of Various Diets

Days on Diet	Weight at Autopsy, gm	Kidney Weight, gm	Kidney to Body Weight Ratio *	Incidence of Sclerosis per 135 Glomeruli
Commercial diet				
151	339	2.4	0.60	38
160	348	2.2	0.63	25
162	269	1.6	0.60	9
185	341	2.3	0.67	21
191	444	2.2	0.59	11
199	452	2.4	0.53	8
199	399	2.4	0.60	22
207	299	2.1	0.70	3
254	380	2.2	0.58	2
261	358	2.4	0.67	15
Crude casein, 200 gm; commercial diet, 800 gm				
156	316	2.6	0.82	10
178	303	2.4	0.72	25
187	290	2.2	0.76	32
192	301	2.3	0.76	18
248	314	3.1	0.99	33
Soybean meal, 200 gm; commercial diet, 800 gm				
159	366	2.8	0.77	37
205	359	3.0	0.84	30
205	457	3.7	0.81	56
205	344	2.3	0.67	15
208	400	3.7	0.92	79
Peanut meal, 200 gm; commercial diet, 800 gm				
171	378	2.5	0.66	21
171	438	2.4	0.55	11
185	426	2.3	0.54	7
185	449	2.3	0.51	11
189	471	3.1	0.65	76

* Kidney ratio; renal weight ÷ body weight × 100.

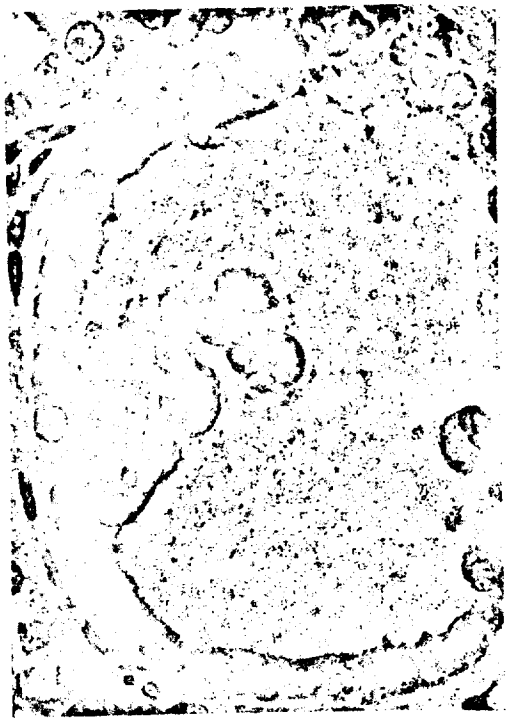

Fig 8.—Some tubules, seemingly reflecting activity of the glomerular epithelium, show hyperplasia of cells and accumulation in "polypoid" structures extending into tubular lumen which contains some granular, cast-like material (hematoxylin-eosin, × 600).

Table 2.—Weighted Means Analysis of Variance of the Means Presented in Tabulation

Source	df	Sum of Squares	MS	F
Total	38	15,578.00		
Treatment	3	2,509.05		
CD vs Ca+S+PM †	1	2,135.14	2,135.14	5.72 *
Among supplemented diets (Ca, S, & PM)	2	373.91	186.96	0.50
Within	35	13,068.95	373.40	

* $P<0.025$.
† CD, commercial diet; Ca, casein; S, soy bean meal; PM, peanut meal.

tuberances extending into tubular lumens (Fig 8). Accumulations of intra-epithelial PAS-positive aggregates were usually associated with degeneration and atrophy of such epithelial cells. Eventual sclerosis of the glomeruli apparently developed without hyperplasia in mesangial and endothelial cells, or evidence of immigration of inflammatory cells (Fig 5 and 7). Following extensive glomerular destruction and sclerosis, hyaline casts became more prominent in the convoluted tubules (Fig 9).

Statistical Analysis of Glomerulosclerosis Counts.—The relationship between glomerulosclerosis and length of time on the diet was tested for significance by linear regression.[12] For the commercial diet, $b = -0.1678$ and $t = 1.29$ with 12 degrees of freedom (df); soybean, $b = 0.4625$ and $t = 1.12$, 7 df; peanut meal, $b = 0.6785$ and $t = 0.379$, 4 df. None of the t values approached the 5% level of significance, and thus no relationship between time on diets and extent of glomerulosclerosis over the ranges studied was indicated. Therefore, the differences in time on the diet for the groups were ignored in the analysis of variance for the glomerulosclerosis counts. The following tabulation shows the means (±SE)

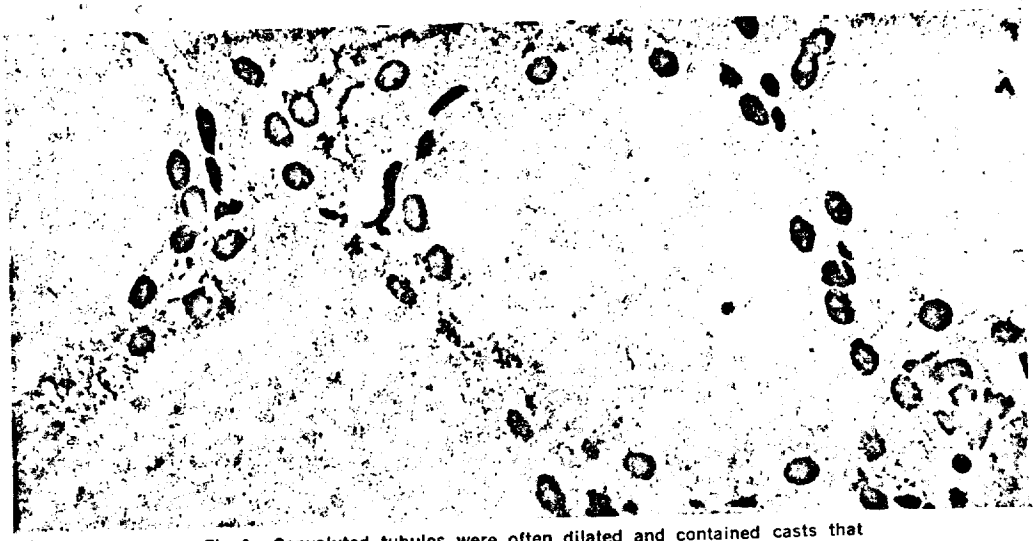

Fig 9.—Convoluted tubules were often dilated and contained casts that were eosinophilic but of otherwise unknown composition (hematoxylin-eosin, × 600).

of the glomerulosclerosis counts from all uninephrectomized rats according to diet:

	$\bar{X}$ (±SE *)
Commercial diet	15.40 (±6.11)
Casein	29.64 (±5.16)
Soybean	37.67 (±6.44)
Peanut meal	30.67 (±7.89)

* Standard error $= \sqrt{\frac{\text{within mean square (MS)}}{n}}$

In Table 2, one observes the results of weighted means analysis of variance of the means presented in the tabulation. The commercial diet group was compared to the average of the casein, soybean, and peanut meal group by the weighted means orthogonal individual degrees of freedom technique.[13] The obtained F value was 5.72 ($P<0.025$). This indicates that the average glomerulosclerosis counts of the three protein-supplemented diets is significantly different from the average observed in the commercial diet. Since the F test for the commercial diet vs the average of other diets was significant, t-tests were done comparing commercial diet to each of the supplemented diets (commercial diet vs casein, $t = 1.78$; commercial diet vs soybean, $t = 2.52$ [$P<0.025$]; commercial diet vs peanut, $t = 1.53$). Only the t-test for soybean vs commercial diet was significant; the other two failed to reach the 0.05 level of significance. On the basis of the obtained mean difference and the within-mean-square value from Table 2 calculations, it is probable that 15 rats per group would have produced a statistically significant difference for each of the protein supplements when compared with the commercial diet. Furthermore, the fact that the F test in Table 2 shows no significant difference among the protein supplements themselves lends additional support to the probability that all of these protein supplements may be equally effective in producing glomerulosclerosis in uninephrectomized rats.

Comment

Our findings support previous observations which suggest that excess protein in the diet somehow induces degeneration of glomeruli and tubules in the rat kidney.[3-7] Uninephrectomized rats have been shown to develop glomerulosclerosis more rapidly than normal animals.[9,10] Accordingly, rats herein described having more than one kidney removed developed glomerular lesions more rapidly than did uninephrectomized rats. Renal degenera-

tion observed in this study and others suggests the thesis that the number of functioning nephrons exerts an appreciable influence upon the development of glomerulosclerosis and tubular degeneration following the ingestion of protein.[11,14] In accord with previous observations, the severity of the protein-nephropathy appears to be affected by the quality of protein because only soybean differed significantly (*t*-test) from commercial diet.[4]

Bras and Ross[3] fed diets containing 30% or 50% protein for 600 days before significant glomerulonephrosis could be observed in rats. They attributed the development of progressive glomerulonephrosis to the combined effects of carbohydrate and protein in the diet. To produce nephropathy with both kidneys present in shorter time intervals than 600 days, diets containing 70% or more protein have been fed. In uninephrectomized rats, such excessive concentrations of dietary protein are not necessary. Accelerated glomerular and tubular deterioration was seen after uninephrectomy even following the feeding of a commercial diet with an estimated 25% protein concentration. In this study, by combining uninephrectomy with the feeding of an estimated 40% protein, appreciable glomerular and tubular degeneration appeared within 150 days. In addition, one can also demonstrate excess quantities of lipid and hemosiderin in kidneys with manifestations of moderate or severe glomerulosclerosis. Whether such accumulations of iron and lipid contribute to or are a complication of the developing glomerular and tubular degeneration is not apparent from these studies. Future investigation will have to resolve whether the iron or the lipid material is responsible for the tan discoloration of the kidneys.

Amino-acid toxicity or imbalance would appear to be a possible explanation for the induction of renal injury for several reasons. It has been shown following intraperitoneal injection that only some amino acids induce proteinuria and hydropic degeneration of the proximal convoluted tubules.[15] Growth of rats fed a diet nearly adequate in protein can be suppressed by amino acid supplements.[16] Supplements of dl-methionine and dl-tryptophan were found to protect rats from induction of acute renal failure following the feeding of gelatin, corn gluten and casein.[8] It is well established that the type of protein ingested exerts an appreciable influence on the amino acid concentration in the plasma[17,18] and excretion in urine.[19] For these reasons, it is believed that ingestion of excess protein could induce a sustained amino-aciduria and cortical degeneration. Proteins of unknown composition have been employed when 20% supplements of casein, soybean, or peanut meal were added to the commercial diet. Nevertheless, it is reasonable to assume that the addition of these proteins did not dilute any essential nutrient in the commercial diet below a critical level because the rats grew satisfactorily when they were fed protein-supplemented diets.

Observations of such renal response to supplemental protein feeding in rodents raise the question of whether glomerular alterations of this type might occur under other experimental conditions. These histologic alterations in rats are not unique to protein overfeeding, because somewhat similar deterioration of glomeruli and tubules has been reported following the production of diabetes by alloxan,[20] prolonged injections of adrenocorticoids,[21] and x-irradiation.[22] Interestingly, in all of these experimental models, a disturbance of nitrogen metabolism may be present.

The major site of alteration remains the (visceral) epithelial cell which shows early evidence of reactive hyperplasia and later disappears, leaving an adhesive contact between extracellular (basement membrane) components of capillary loops and Bowman's capsule. In rats surviving five sixths nephrectomy for periods rang-

ing from 10 to 50 weeks, ultrastructural studies have revealed accumulations of granular osmiophilic material, fusion of foot processes, and vacuolization of epithelial cells.[23]

In this study, the epithelial reaction is not confined to two glomerular layers, but also appears in proximal and distal convolutions. Alterations that have been recognized in the tubules by light microscopy are hyaline droplet formation, hyperplasia of epithelium, and cast formation. Intracellular aggregation of PAS-positive material was more prominent in epithelial cells of tubules when glomerular degeneration was more extensive. It is possible that these hyaline droplets represent cytosomal accumulation of protein, reflecting increased glomerular passage of serum proteins. The hyperplasia of tubular epithelium seen in these experiments may be of considerable theoretical interest because there are relatively few clinical entities in which this occurs.

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Effect of Factors other than Choline on Liver Fat Deposition.* (19904)

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The effects of certain amino acids on the metabolic relationship between tryptophan and niacin have been studied extensively (1-6). Recently we were surprised to observe fat deposition in the livers of animals receiving similar diets to those used in the above studies. Since the basal ration contained 0.1% choline and since methionine failed to prevent the occurrence of fatty livers, it appeared that other factors in the ration were involved.

In this paper, we wish to present experiments which demonstrate certain effects of proteins, amino acids and carbohydrate on liver fat deposition.

Experimental. Three-week-old male Sprague-Dawley rats kept in individual cages

TABLE I. Basal Ration for Fat Deposition Studies.

Component	Level in diet (per 100 g ration)
Casein	9 g
L-cystine	.2
Sucrose or dextrin	81.9
Corn oil	5
Salts IV(7)	4
Thiamin · HCl	.2 mg
Riboflavin	.3
Pyridoxine · HCl	.25
Ca pantothenate	2
Choline chloride	100
Inositol	10
Biotin	.01
Folic acid	.02

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TABLE II. Effect of Various Supplements to Sucrose Basal Ration on Liver Fat and Growth Rate.

Supplement	No. of animals	50 mg DL-tryptophan	1.5 mg niacin	% fat in rat livers — Wet basis Avg	Wet basis Range	Dry basis Avg	Dry basis Range	Avg growth rate (g/wk) Avg	Avg growth rate (g/wk) Range
0	9			8.8	(4.4-13.3)	20.4	(12.7-28.4)	14.4	(10.4-20.8)
0	9	+		9.3	(6.6-15.9)	23	(13.5-37.8)	19.8	(15.2-26.4)
0	9		+	13.4	(9.5-19.9)	32.3	(24.4-41.7)	16.4	(8.8-20.8)
6% gelatin	6			2.9	(2.2- 3.8)	8.3	(6.2-10)	3.2	(1 - 6.6)
	6	+		4.8	(3 - 9.6)	11.9	(8.4-15.2)	25.6	(19.2-35)
	6		+	3.7	(3 - 4.4)	11.1	(8.9-14)	25.6	(14.2-38.8)
.16% threonine	9			3.2	(2.3- 4.7)	8.7	(6.5-10.1)	4.4	(.8- 7.6)
	9	+		6.6	(3.8-13.5)	17.5	(11.3-26.6)	23	(14.8-29)
	9		+	7.3	(5.1-15.1)	19.7	(15.1-35.5)	20	(12.2-35.2)
.31% threonine	6			2.4	(2.3- 2.5)	6.9	(6.4- 7.4)	3.2	(2.2- 4)
	6	+		6.2	(5.9- 6.9)	16.1	(15 -18.1)	22	(21.8-22.4)
	6		+	6.5	(5.7- 7.9)	17.2	(15 -19.9)	22.8	(19.2-26.2)

were fed *ad libitum* and were weighed at weekly intervals. The basal diet to which supplements were made at the expense of the carbohydrate is shown in Table I. This basal diet contained cystine in all cases except where indicated. The fat-soluble vitamins were administered orally in the form of 2 drops of halibut liver oil each week. The animals were sacrificed at the end of a 5-week period and the livers of the animals were removed and analyzed for fat content using essentially the procedure outlined by the Association of Official Agricultural Chemists(8). The livers were dried at 50°C in a vacuum oven for moisture determination, and the fat content was determined on duplicate samples of the dried livers. Samples were ground with sand and extracted with ether for 16 hours on Goldfisch extractors. After evaporation of the ether the fat samples were heated in a 100°C oven for 15 minutes, cooled in a desiccator, and weighed to constant values.

Results. The data in Table II show the effect of various supplements when added to sucrose rations containing 9% casein with 0.2% L cystine. When either 50 mg % DL tryptophan or 1.5 mg % niacin was added to this basal ration the growth rate increased but no protective effect upon liver fat deposition was noted. When 6% gelatin was added to the basal ration, however, a decided depression of liver fat together with a reduced growth rate occurred. Either tryptophan or niacin, added with the 6% gelatin supplement, stimulated growth over the controls while the liver fat was still significantly lowered. From this experiment, it appeared that the factors controlling growth and liver fat deposition were not the same. When 0.15% DL threonine was added to the basal ration, growth was inhibited as much as that caused by 6% gelatin and the liver fat was lowered to the same level. When the indicated amounts of either tryptophan or niacin were added to the threonine supplement, growth was increased markedly, but no depression of liver fat occurred. The growth depression was undoubtedly due to an amino acid imbalance(4). Either niacin or tryptophan alleviated this growth depression but an increase in liver fat still occurred.

Griffith and Nawrocki(9) have reported intensification of a choline deficiency when threonine was added with cystine to a low casein diet. The influence of threonine was believed to be due to a direct stimulation of growth or metabolism rather than an antagonistic action towards labile methyl. This seems to be the case since 0.1% choline was present in the basal ration under our conditions. The growth depression which we observed is probably due to a tryptophan-niacin deficiency, yet when either of these factors was added to bring about a growth response, fatty livers occurred. When the level of threonine was doubled as in the last experiment in Table II in the presence of either tryptophan or niacin, growth was restored and liver lipids were reduced, but not to the extent brought about by the gelatin supplement

TABLE III. Effect of Various Supplements to Dextrin Basal Ration on Liver Fat and Growth Rate.

Supplements	No. of animals	50 mg DL-tryptophan	1.5 mg niacin	% fat in rat livers — Wet basis Avg	Wet basis Range	Dry basis Avg	Dry basis Range	Avg growth rate (g/wk) Avg	Avg growth rate (g/wk) Range
0	6			4.9	(3.7-6)	13.9	(11.2-17.6)	21.8	(14 -26.8)
0	6	+		3.9	(3.2-5.2)	11.4	(9.4-14.7)	17.8	(9.2-26.2)
0	6		+	5.9	(5.3-6.5)	16.3	(14.2-18.5)	22.4	(16.2-26)
6% gelatin	6			3.1	(2.5-4.1)	9.3	(7.2-12.5)	7.6	(6 - 9)
	6	+		4.2	(3.5-4.7)	12.9	(11.2-14.1)	22.8	(14.8-30.4)
	6		+	3.2	(2.4-4.4)	9.6	(8 -12.2)	22	(12.8-32.6)
.18% threonine	3			3.9	(3.3-5)	8.9	(7.7-10.7)	11	(5.4-15.6)
+.24% phenyl-	3	+		3.1	(2.4-4.1)	8.9	(7.2-11.3)	31.4	(10.4-32.4)
alanine	8		+	4.5	(4.1-5.3)	13.5	(12.6-15.2)	23.6	(18.8-26)
2% acid-hydro-	3			4.3	(2.9-6.4)	12.8	(8.8-18.8)	15	(13.6-17.6)
lized casein+	3	+		5.1	(4 -5.8)	15.1	(12.2-17.6)	30	(27.6-33.8)
1.5% glycine	3		+	5.6	(5.4-5.7)	16.8	(16.5-17.1)	23.4	(19.2-27.8)

alone. It is clear that gelatin exerts an effect beyond the action of threonine.

In Table III similar data are given when dextrin rather than sucrose was the carbohydrate. The controls on the dextrin basal showed a higher growth rate even without additions of niacin or tryptophan. At the same time it can be noted that the deposition of liver fat was not as severe as in the sucrose group. The addition of either tryptophan or niacin in the indicated quantities failed to promote increased growth over the controls. When 6% gelatin was added to the basal diet, a drop in liver lipids and growth rate was observed. Supplements of either tryptophan or niacin together with gelatin completely overcame the apparent growth antagonism precipitated by the gelatin and maintained the liver fat at low levels. When 0.18% DL threonine and 0.24% DL phenylalanine were supplemented to the basal ration, a similar effect was noted. The addition of either tryptophan or niacin restored the growth without affecting the liver fat. In the final experiment in Table III, 2% acid-hydrolyzed casein and 1.5% glycine were added together to the basal ration. The growth was depressed but liver fat was only slightly altered if at all with respect to the controls. Tryptophan or niacin increased growth and liver fat deposition slightly. The dextrin basal rations supported growth rates of control animals which were much greater than the sucrose controls as has been previously observed in this laboratory by Teply and coworkers(10). On the other hand, it can be observed that the lipid contents of the livers of the dextrin controls were lower. Gelatin exerted a greater effect when sucrose was the carbohydrate.

McHenry(11) cited the roles of biotin, thiamin and carbohydrate in the production of fatty livers. More recently Harper and Katayama(12) have studied the utilization of sucrose or corn starch for growth when added to low casein diets. They showed that corn starch supported better growth than sucrose in a 9% casein basal ration supplemented with methionine. This was also true when methionine was not added to the basal diet. One can surmise that the differences between the 2 carbohydrates may be in the rate of passage of the diet through the gut. A greater availability of the amino acids in the casein might be suspected if the ration passed through the intestine more slowly. The observations by Mulford and Griffith(13), and Salmon(14) that cystine supplementation aggravates renal damage and fatty infiltration of the livers of rats receiving 8% casein diets deficient in choline; and Griffith and Nawrocki(9) that further intensification of the choline deficiency occurred when threonine was added with cystine, prompted us to carry out a third set of experiments which are reported in Table IV. We decided to omit cystine from the basal ration and test the effects of certain protein and amino acid supplements. The most striking effect of the cystine omission may be seen in the control group where the growth was about 50% lower

TABLE IV. Effect of Increasing Protein and Single Amino Acids Supplemented to Sucrose Basal Ration Without Cystine.

Supplement, %	No. of animals	% fat of dry liver		Avg growth rate (g/wk)	
		Avg	Range	Avg	Range
0	6	29	(13.9-35.2)	7.6	(5.8-11.6)
3 Casein	6	11.5	(6.9-17)	15.6	(12.6-18.8)
6	6	10.2	(7 -15.2)	22.6	(16.2-24.8)
3 Gelatin	6	17.2	(9.7-25.8)	10.4	(8.8-12.2)
6	6	13.9	(9 -18.4)	10	(6.2-13.8)
.3 L Proline	6	23.6	(14.8-35.8)	6.6	(1.8- 9.2)
.6	6	24.5	(17 -25.4)	7.2	(4.8-10.4)
.6 DL Alanine	6	23.1	(15.6-28.1)	5.6	(4.2- 8.4)
.3 DL Methionine	3	26.7	(23.6-29.6)	14.9	(10 -17.6)

than similar controls in Table II where cystine was included. It is likely that the sulfur amino acids were limiting for growth in the 9% casein rations(4). Larger amounts of casein added to the basal diet increased growth and decreased liver fat. This, however, was not true in the case of gelatin. Increasing levels of gelatin failed to stimulate growth proportionately to the level added to the diet, but did decrease liver fat proportionately. L proline was chosen as an amino acid supplement because of its high concentration in gelatin and DL alanine was chosen because it showed a slight effect upon the reduction of liver lipids in preliminary trials. The supplements of 0.3% L proline and 0.6% DL alanine are comparable and each decreases the liver fat slightly while the growth in either group is similar to the controls. This observation strengthens the earlier conclusion that those factors controlling the liver fat deposition are not necessarily the same as those controlling growth. To show this more clearly, 0.3% DL methionine was added to the basal ration which resulted in a restoration in the growth equivalent to that exerted by cystine in the control group presented in Table II. While the addition of methionine to the basal ration without cystine increased growth 100% over the controls, it exerted no effect upon the liver fat deposition. It appears that 0.3% DL methionine improved growth without antagonizing liver fat deposition as is often the case when added cystine is present(9,13-15).

The fatty liver which we have obtained under our conditions seems to be one which may be influenced by at least 3 factors: protein, amino acids and carbohydrate. There has been much conjecture as McHenry(11) has pointed out, upon the role of the pancreas in preventing fatty livers. Some observations suggest the essential factor in the pancreas to be the hormone, lipocaic, while other workers claim to have ruled out the hormone action and assert the idea that the proteolytic enzymes from pancreatic juice are necessary for the complete digestion of dietary protein which then reduces fat deposition by some mechanism; perhaps one which involves one or more amino acids specifically. It seems likely from our data that, under our conditions, carbohydrate may effect liver fat and growth by controlling the rate of absorption of the necessary amino acids from the gut by affecting the rate of passage of the diet through the gut or affecting the microorganisms for production of necessary vitamins, or both. Whole protein, such as casein or gelatin, apparently supplies necessary amino acids for controlling liver fat deposition. While certain individual amino acids exert a slight effect on the reduction of liver lipids, it appears that combinations of these amino acids are necessary to bring about a greater reduction in liver fat deposition. (Harper, Monson, Elvehjem work in progress.)

Summary. A number of factors under our experimental conditions influence fat deposition in the liver: the availability of niacin to the animal; the type of carbohydrate used in the ration and type and level of protein used. Individual amino acids and mixtures of amino acids which support optimal growth conditions do not produce minimum levels of fat

in the liver.

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THE DIGESTION AND ABSORPTION OF P^{32} LABELED CASEIN BY THE YOUNG CALF [1]

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THREE FIGURES

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Much reliable information can be found on the utilization of casein by the laboratory rat. On the other hand, very little data are available on the utilization of this important protein by the young calf. With the recent improvement in purified liquid diets (Wiese et al., '47) and the increasing availability of radioactive isotopes, there existed an opportunity to obtain some desired information as to the extent and mechanism of utilization of casein by the calf. The radioactive isotopes of nitrogen have half-lives of such short duration that they are of little value as tracers in studies of this sort. Casein, however, is a phosphoprotein and can be labeled with P^{32}, a radioactive isotope of phosphorus having a convenient half-life of 14 days (Kleiber et al., '48). Using casein labeled with P^{32}, research was begun to determine: (1) the rate of digestion of casein as measured by the appearance of the isotope in the blood stream; (2) the fate of the phosphorus present in the casein molecule during the process of digestion; (3) the distribution of isotopic phosphorus in the tissues of the calf's body; (4) the extent of digestion of the casein; and (5) the metabolic fecal protein excretion of the

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young calf under conditions of milk feeding. A preliminary report of this work has been presented (Lofgreen, '50). The present paper presents more detailed results on the first three objectives. Data on the latter two will be presented at a later date.

EXPERIMENTAL

Casein labeled with P^{32} was prepared by injecting lactating cows with from 40 to 100 millicuries of P^{32} as sodium phosphate in an isotonic saline solution. Injections were usually divided into 4 or 5 equal portions and made at 12-hour intervals. Milk was collected beginning after the second or third injection and continuing until such time as a sufficient quantity was obtained to yield the desired amount of casein. One part of skim milk was diluted with 4 parts of water and 0.1 part of 6% acetic acid was added with stirring. The precipitated casein was washed successively with water, alcohol and ether and allowed to dry. The casein prepared in this manner was incorporated into a purified diet similar to that described by Wiese et al. ('47).

Four calves between 9 and 10 weeks of age were given a single feeding of the diet made with the labeled casein. By means of venous catheterization similar to the method described by Ralston et al. ('49), blood samples were taken at frequent intervals following feeding. At 24 hours after feeding two of the calves were sacrificed and samples were taken for tissue distribution studies. For comparative purposes two similar calves were fed inorganic phosphate solution containing P^{32} by adding it to their milk for a single feeding. Blood samples were taken and the calves sacrificed 24 hours later to determine tissue distribution. Two more calves were injected every 20 minutes over a period of 24 hours with graded doses of inorganic phosphate solution containing P^{32} in an effort to duplicate as closely as possible the blood levels of P^{32} obtained by feeding labeled casein. Injections were made into one jugular vein and blood samples withdrawn from the other to avoid contamination. Blood and

tissue samples were taken as in the case of the other calves. A summary of data concerning the animals used and their treatment is presented in table 1.

All samples were dried, mixed with magnesium nitrate and ashed at 500°C. The ash was taken up in dilute HCl, keeping the volume small in order to maintain radioactivity at as high a level as possible. Radioactivity was determined by use of a Geiger counter and total phosphorus by the method of Fiske and Subbarow ('25). Because of the wide differences in dosages and body weights of the calves, the absolute blood and tissue levels were not directly comparable. Radioactive phosphorus content is therefore expressed herein as corrected specific activity, which is equal to the microcuries (μc) of P^{32} per gram of phosphorus divided by the dose (in μc) per 100 gm body weight.

TABLE 1

Description and treatment of experimental animals

CALF NO.	BREED[1]	AGE	WEIGHT	P^{32} ADMINISTERED	
				AMOUNT	METHOD
		days	*kg*	*μc*	
3	J	71	42.2	293	Fed in labeled casein
4	J	56	48.1	2760	Fed as inorganic salt
5	H	63	57.2	26.5	Fed in labeled casein
6	H	67	78.5	260	Injected as inorganic salt
7	H	68	68.5	72	Fed in labeled casein
8	G	63	54.5	60	Fed in labeled casein
9	H	67	73.5	1000	Fed as inorganic salt
10	G	67	45.4	1000	Injected as inorganic salt

[1] Guernsey, Holstein and Jersey are abbreviated as G, H and J, respectively.

RESULTS AND DISCUSSION

The blood levels of radioactive phosphorus for 24 hours following feeding are presented in figures 1 and 2. Kleiber et al. ('50a, b) have shown that there is a continuous removal of labeled phosphate from the blood stream of cows. The rate of removal is dependent upon the level in the blood.

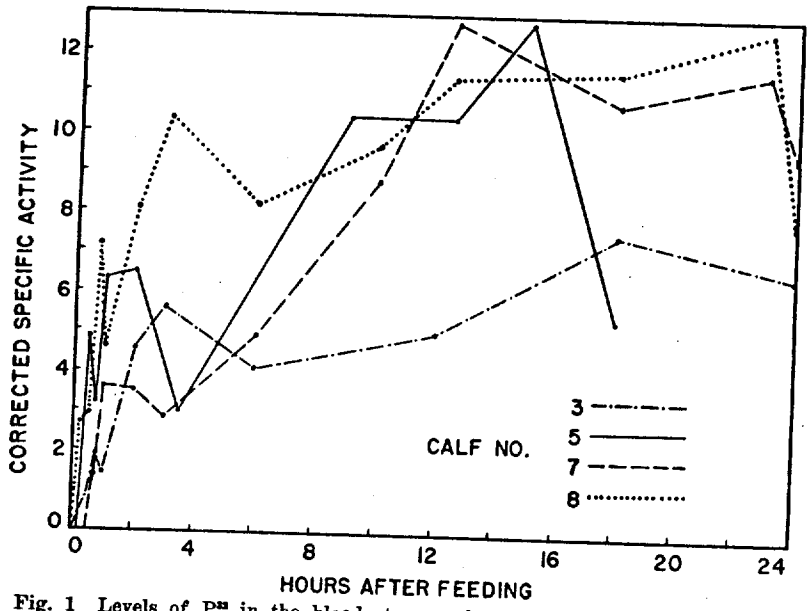

Fig. 1 Levels of P^{32} in the blood stream of calves fed labeled casein.

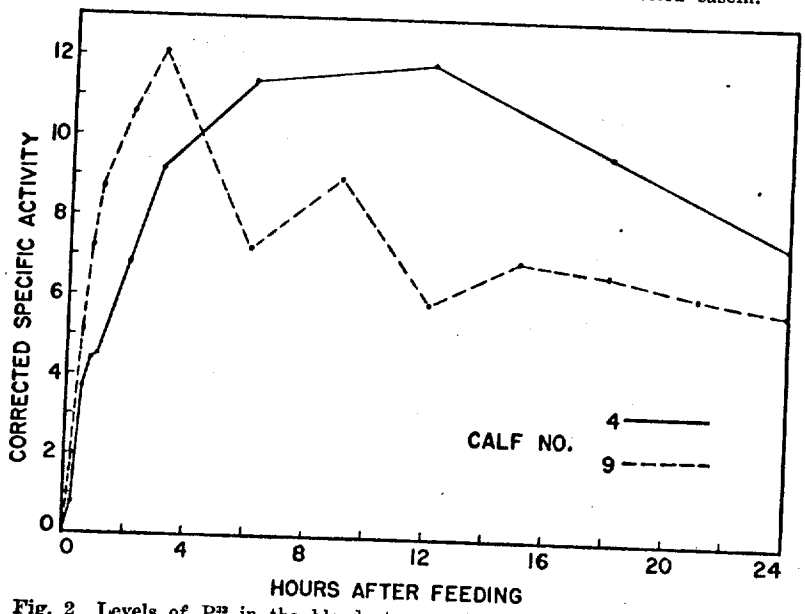

Fig. 2 Levels of P^{32} in the blood stream of calves fed inorganic phosphate in milk.

Changes which occur in the corrected specific activity in the blood of the calves fed P^{32} either in the form of casein or as inorganic phosphate would therefore reflect changes in absorption rate. For example, when the specific activity of the blood remains constant or increases despite this withdrawal, one must conclude that absorption is taking place at correspondingly different rates.

There is some variation in the curves representing the absorption of P^{32} from the labeled casein. There are, however, certain similarities. All 4 curves show an initial rise to a peak in from one to three hours, a fall and a more gradual rise to a second peak at from 12.5 to 23 hours. In two of the calves measurable blood levels of P^{32} were reached as early as 15 minutes after feeding. Animal phosphatases will not attack the phosphorus-amino acid linkage to any great extent in the intact casein molecule (Kay, '34). It appears, therefore, that within 15 minutes the proteinases and peptidases of the gastric, pancreatic and intestinal secretions may hydrolyze the casein molecule to the point where either the phosphate-containing substances can be absorbed or the phosphatases can attack the phosphate-amino acid linkage, releasing inorganic phosphate which is then absorbed. Even though the digestion process is initiated rapidly it continues for a long period, as is shown by the time at which the maximum levels are reached. This is in contrast to fat absorption by the calf, which reaches a peak during the first 6 hours and decreases to its initial level by the 12th hour (Barker and Jacobson, '49).

The blood levels of P^{32} presented in figure 2, obtained from the calves fed inorganic phosphate in their milk, are markedly different from those of the animals fed casein. This is as would be expected, since no digestion is necessary before the phosphate can be absorbed. It might be suspected from a comparison of the curves in figures 1 and 2 that the rather rapid rise to the first peak in the curves of the calves fed casein may have been due to the absorption of inorganic phosphate which had not been washed from the labeled casein

as it was prepared. In order to investigate this possibility the true protein was determined in a sample of casein by the method of Barstein (Wiegner, '26). The original casein contained 0.346 μc of P^{32} per gram, while the true protein prepared from 1 gm of casein contained 0.343 μc, showing that practically all the activity had been retained in the true protein fraction. It therefore seems reasonable to conclude that there was little contamination of the casein by inorganic phosphate and that the absorption curves in figure 1 represent the absorption of phosphorus which had been organically bound in the labeled casein.

During the process of digestion of the casein molecule there is undoubtedly some liberation of the phosphorus-containing compounds, of which phosphoserine may be the predominating one (Kay, '34). Damodaran and Ramachandran ('40) have presented evidence that an enzyme-resistant phosphopeptone containing glutamic acid, isoleucine and serine can be isolated from casein by subjecting it to the action of pepsin and trypsin. The possibility exists that some of these compounds may be absorbed unchanged into the blood stream. If significant amounts of phosphoserine or phosphopeptone are absorbed with the phosphorus remaining organically bound, it seems logical to expect that the tissue distribution of P^{32} in calves fed casein would be quite different from the distribution in those absorbing or having injected directly into the blood stream the phosphate in the inorganic form. A comparison of the tissue distribution of P^{32} between calves having similar blood levels over a 24-hour period but with the P^{32} coming in one case from injected P^{32} and in another case from digested casein should give some information concerning the absorption of the phosphorus in organic or inorganic form. Such a comparison is presented in figure 3. The corrected specific activities for the tissues of the calves fed either casein or inorganic phosphate are, with the exception of the organs of the gastrointestinal tract, consistently lower than those of the injected calves because of the incomplete absorption from the digestive tract. The specific activity values have been

corrected for the total dose per unit of body weight, while the dose actually reaching the tissues of the calves fed casein or inorganic phosphate would be somewhat less than the total

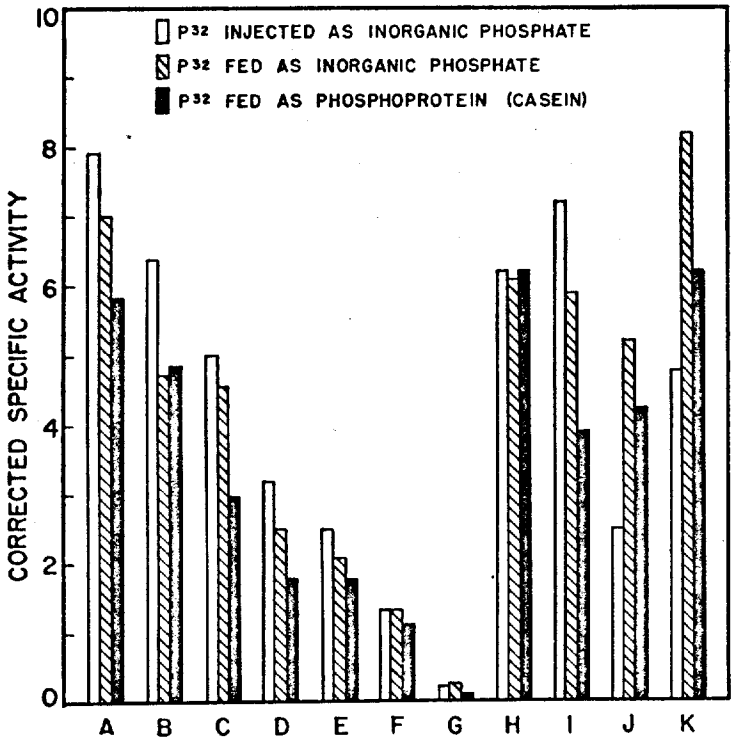

Fig. 3 Distribution of P^{32} in representative tissues of calves fed either labeled casein or labeled inorganic phosphate or injected with labeled inorganic phosphate. A, liver; B, kidney; C, heart; D, lung; E, testes; F, skeletal muscle; G, brain; H, rumen; I, omasum; J, abomasum; K, jejunum.

consumed. If it were possible to correct for the incomplete absorption the levels would be more nearly equal.

The distribution pattern, excluding the tissues of the gastrointestinal tract, is very similar among the three methods of administration. Since it would be logical to expect a different pattern of distribution of phosphorus organically

bound to an amino acid and inorganic phosphate not so bound, the results of this study give no evidence that phosphoserine or phosphopeptone are absorbed as such. The liver showed the highest rate of phosphate exchange in all except one calf, in which the exchange rate of the kidney equaled that of the liver. The brain consistently showed a very slow rate of exchange.

The pattern of distribution in the organs of the gastrointestinal tract seems to be different in that it appears to depend upon the method of administration. It is quite difficult to wash the organs free of contamination when they have been directly in contact with either the inorganic P^{32} or the casein. This would occur with the calves fed casein or inorganic phosphate and could account for the differences in distribution pattern among the three methods of administration. The measurement of the distribution in the organs of the gastrointestinal tract of the injected calves should therefore be more valid than that in the calves fed P^{32} in either form.

The specific activity of the tissue of the abomasum is about one-half that in the jejunum. Since there was a practically continuous supply of P^{32} being injected into the blood stream for 24 hours, and since one must assume that the specific activity before injection was zero, it is probable that the tissues possessing a high specific activity at the termination of injections are those tissues which have a high rate of phosphate exchange. At lengthening periods after cessation of P^{32} administration this assumption may not be true, since the organs with the more rapid exchange rate would lose their P^{32} content more rapidly than those exhibiting a slower rate. Because the calves were sacrificed just at the termination of the injections, it seems justifiable to consider that the tissues having a high specific activity also possess a high rate of phosphate exchange. It appears, therefore, that the abomasum exhibits a much slower rate of phosphate exchange than does the jejunum. There is considerable evidence that rapid phosphorylation and dephosphorylation mechanisms are important

in the absorption of certain nutrients (Somogyi, '42; Deuel, '43; Evans, '44). It is possible that the more rapid rate of phosphate exchange in the small intestine may be due in part to the importance of this organ in absorption processes. In order to determine if the same relationship between the distribution in the tissues of the stomach and the small intestine 24 hours after injection would hold true in other animals, comparisons were made from data available at this institution. A summary of such data is presented in table 2.

The specific activity of the tissue of the small intestine in all cases is almost twice as high as that in the abomasum.

TABLE 2

Specific activity of the tissues of the small intestine and true stomach of different animals sacrificed 24 hours after injection of P^{32}

ANIMAL	μC P^{32} PER GRAM OF P		
	SMALL INTESTINE	TRUE STOMACH	RATIO
Rabbit	260	167	1.6
Hog	295	171	1.7
Sheep	76	47	1.6
Calf	33	18	1.8
Cow	20	10	2.0

In table 3 are presented the ratios of the specific activities of the various organs of the digestive tract to those of the abomasum of the two injected calves, 6 and 10.

In both calves all organs had a higher specific activity, and thus a higher rate of exchange, than the abomasum, with the omasum, rumen and jejunum ranking highest. The rapid rate of exchange in these organs may be due to either phosphorylation mechanisms involved in the absorption of nutrients, phosphorylation processes concerned in the metabolism of the tissue itself, or merely the transport of phosphate from the blood to the gastrointestinal contents. As was previously pointed out, phosphorylation mechanisms may be important in absorption. On the basis of the oxygen consumption of intestinal tissue in comparison to that of the stomach, one

might expect this organ to exhibit a rapid rate of phosphate exchange. Carlyle ('48) reported that the intestine of sheep consistently exhibits a higher Q_{O_2} than the stomach. Dickens and Weil-Malherbe ('41) state that the mucous membrane of the jejunum is among the most actively respiring tissues of the body. The possibility exists that the rapid exchange rates of the omasum and rumen may be partially explained on these bases. Barcroft et al. ('44) have shown the rumen to be important in the absorption of organic acids. Wildt (1874) noted that phosphorus secretion exceeded absorption in the rumen and abomasum, while absorption exceeded secretion in the

TABLE 3

Ratio of the specific activity of the tissues of the gastrointestinal tract to that of the abomasum of calves injected with P^{32}

ORGAN	CALF	
	6	10
Rumen	2.9	1.9
Omasum	2.9	2.8
Jejunum	2.0	1.8
Cecum	1.8	1.2
Colon	1.7	1.6
Rectum	1.5	1.5
Abomasum	1.0	1.0

omasum and small intestine of sheep. It appears doubtful that the transport of phosphate from the blood through the rumen wall to the contents could account for the high activity of the rumen tissue, especially when one considers that the abomasum exhibits a slow rate of exchange and that phosphate is also transferred from blood to contents in this organ. This factor certainly cannot account for the high specific activity of the omasum of the injected calves, since absorption exceeds secretion in this part of the tract. Further work must be done to determine the significance of the phosphate exchange rates with respect to the role of these organs in the absorption process.

SUMMARY

Studies are reported on the rate of absorption of P^{32} by calves fed labeled casein, and comparisons are made with similar calves fed or injected with the isotope in inorganic form. Tissue distribution of the P^{32} was determined 24 hours after feeding.

The results show that the digestion of casein is initiated rather rapidly and is prolonged over the 24-hour period. When labeled inorganic phosphate is added to the milk, absorption takes place in a much different pattern.

Tissue distribution of the isotope indicated that it is probable that the calf absorbs inorganic phosphate even when it is fed in the organic form as phosphoprotein. Liver and kidney consistently showed rapid phosphate exchange rates, while brain tissue rates were notably slow. The omasum, rumen and jejunum appeared to have high phosphate exchange rates in comparison to other sections of the gastrointestinal tract.

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Effect of Methionine and Casein on Acetoacetate Induced Hyperglycemia. (21605)

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Lazarow(1,2) reported that injection of large doses of glutathione immediately preceding a diabetogenic dose of alloxan completely protects rats from diabetes. Griffiths (3) showed that diabetes can be induced in rabbits by injection of uric acid only when the blood glutathione level is depleted by keeping the animals on a cystine-methionine deficient diet. He observed further that if the deficient diet was supplemented by 0.2% methionine the blood glutathione level did not fall appreciably nor did the rabbits develop hyperglycemia following uric acid injection. Nath and his associates(4,5) reported that gradual accumulation of the acetoacetate in the system is responsible not only for the on-

set of hyperglycemia but also for the development of other diabetic symptoms associated with decreased sugar tolerance in the experimental animals (rabbits) kept on Bengal gram (*Cicer areatinum*) diet. This fat metabolite has further been shown in this laboratory to cause inactivation of endogenous insulin both *in vivo* as well as *in vitro* and to bring about considerable decrease in the potency of pancreatic insulin of guinea pigs on prolonged injection(6,7), also to bring about a marked depletion in blood GSH of these animals(8) and to increase their susceptibility to alloxan diabetes(9).

Tidwell *et al.*(10) have reported that they could not find any appreciable effect of sodium acetoacetate on glycogen storage in the liver and muscle of rats, neither could they observe any change in blood sugar except slight hypoglycemia(11). Since they used a different species of animals and a diet, in which the percentage of protein (casein) was higher than that of ours and since casein contains an appreciable amount of methionine, it was thought worthwhile to investigate the role of methionine and casein on acetoacetate induced hyperglycemia in rabbits with a view to account for the difference in behavior shown by the two different types of animals and those again kept on different diets.

Experimental. Forty healthy rabbits each weighing approximately 2 kg body weight were divided into 6 groups. The animals of Group I were kept on the cystine-methionine deficient diet prepared according to Griffiths(3). The animals of Groups II, III, IV, and V were fed cystine-methionine deficient diet supplemented with varying amounts of methionine as shown in Table I. The last group of animals were kept on cystine-methionine deficient diet in which 26% carbohydrate was replaced isocalorically by 26% casein. All the animals were kept in individual cages. Sodium acetoacetate was given intraperitoneally in increasing doses beginning from 50 mg/kg body weight. Blood sugar was determined periodically according to the method of Hagedorn and Jensen(12) and blood GSH was estimated by the method of Woodward and Fry(13). The results are shown in Tables I and II.

Results. Data as per Table I indicate that

TABLE I. Effect of Methionine and Casein on Acetoacetate Induced Hyperglycemia.

No. of rabbits	Nature of diet	Blood sugar, mg/100 cc, after dose of injection*					
		Initial	10	30	40	55	70
10	SH deficient	108.2 ± 4.5	114.7 ± 8.2	162 ± 10.2	172.9 ± 12.5	178.6 ± 10.5	183 ± 12.8
5	*Idem* + 0.2% methionine	108.8 ± 3.8	112.1 ± 4.8	160 ± 10.5	170 ± 8.5	173 ± 12.5	180.3 ± 15.5
5	" + 0.5% "	109 ± 4.8	115.8 ± 2.5	152.6 ± 12.8	170 ± 5.8	170.6 ± 5.2	179.6 ± 8.2
5	" + 1 % "	108.3 ± 4.2	109.6 ± 3.8	110.3 ± 4.2	112.6 ± 5.8	116 ± 4.0	124.3 ± 6.2
5	" + 2 % "	108.6 ± 3.8	110 ± 2.5	112 ± 2.2	110.8 ± 3.8	111 ± 3.2	112.4 ± 4.0
4	" + 26 % casein	108.3 ± 3.8	110.3 ± 3.2	111.2 ± 3.2	110.8 ± 3.8	112.8 ± 5.8	124.5 ± 7.5

* Daily dose of acetoacetate injection: 1st to 10th day, 50 mg/kg; 11th to 30th day, 100 mg/kg; 31st to 55th day, 150 mg/kg; 51st to 70th day, 225 mg/kg.

TABLE II. Effect of Methionine and Casein on the Blood GSH of the Animals (Rabbits) Injected with Acetoacetate.

No. of rabbits	Nature of diet	Blood GSH in mg/100 cc blood after days of injection					
		Initial	10	30	40	55	70
12	SH deficient	31.7 ± 1.9	25.1 ± 1.9	20.4 ± 1.4	18.6 ± 1.8	16.2 ± 1.6	14.5 ± 1.8
6	*Idem* + .2% methionine	32.4 ± 2.1	29.0 ± 2.6	21.5 ± 2.0	18.6 ± 2.4	16.3 ± 2.2	14.6 ± 2.2
6	" + .5% "	31.7 ± 2.5	29.3 ± 2.3	22.8 ± 1.6	20.5 ± 2.5	16.4 ± 2.2	15.5 ± 2.0
3	" + 1 % "	30.9 ± 1.2	30.3 ± 1.0	26.2 ± 1.3	25.0 ± 2.0	22.0 ± 2.2	20.8 ± 2.7
6	" + 2 % "	31.4 ± 1.7	30.5 ± 2.7	30.2 ± 2.2	29.9 ± 2.0	27.9 ± 1.7	27.1 ± 1.9
4	" + 26 % casein	31.7 ± 2.2	31.0 ± 1.9	29.8 ± 2.6	28.2 ± 2.1	24.3 ± 3.2	21.8 ± 2.4

methionine in large concentration leads to the prevention of hyperglycemia caused by daily repeated injection of acetoacetate. Methionine, which when given in lower concentration, *i.e.*, 0.2% to 0.5% of the diet, shows but slight effect on the prevention of hyperglycemia, is highly effective while used in higher concentration (*i.e.* 1%) up to a period of 40 days. If the concentration of methionine is increased still further (*i.e.* 2%) it can completely protect the animals from hyperglycemia throughout the period of experiment (*i.e.* 70 days) accompanied with only a slight fall in blood GSH. It can also be seen from Table I that animals maintained on the diet supplemented with casein (26%), as used by Tidwell *et al.*(11), show normal blood sugar values up to 40 days; but as the injection is continued for another 30 days, there is a slight tendency of hyperglycemia in these animals. Such hyperglycemia seems to be due to the insufficiency of methionine owing to the increased demand of such compounds, through the continuous injection of acetoacetate, as is evident from the fact that the animals supplemented with 2% methionine show no indication of the development of hyperglycemia at all.

The data further show that there is an inverse relationship between the blood sugar level and the glutathione concentration of blood. The greater the depletion of blood GSH, the greater is the value of blood sugar. Similar behavior has also been reported earlier from this laboratory(9). The animals, whose blood GSH concentration is maintained at the normal level by supplementing methionine in the diet, do not exhibit hyperglycemia thus showing that glutathione plays an important part in the prevention of acetoacetate induced hyperglycemia and the addition of the requisite amounts of methionine or casein protects the animals from the depletion of blood GSH.

Tidwell *et al.*(11) failed to observe hyperglycemia in rats kept on a diet containing 26% of casein through acetoacetate injection. We have also not observed any appreciable change in blood sugar of rabbits kept on such diet for 40 days. In a recent communication from this laboratory(14) it has been shown that rats require very high concentration of acetoacetate for producing hyperglycemia and glucosuria. In a previous communication, it was shown by Nath and Chakrabarti that β-hydroxybutyrate in lower concentration can deplete liver and muscle glycogen in rats on 20% casein diet(15). This difference in behavior of the two substances in rats was accounted for through the difference in their capacity for biosynthesis of ascorbic acid, which has been found to be stimulated in rats by sodium acetoacetate injection. But this biosynthesis of ascorbic acid was found to be retarded by acetoacetate in higher concentration. We observed hyperglycemia and depletion of glycogen storage in liver and muscle of rats by injecting sodium acetoacetate in higher concentration. The higher percentage of casein in the diet, which Tidwell *et al.* used in their experiments might account for their failure to notice hyperglycemia in rats by acetoacetate injection. Deficiency of SH compounds seems to be an essential factor for acetoacetate induced diabetes.

Further, it has recently been reported by Chari and Wertheimer(16), who have indicated clearly from their studies with rat diaphragm that acetoacetate at a concentration higher than that of glucose has a specific action on the retardation of glycogen synthesis even in presence of insulin; but while the concentration of acetoacetate is much lower than that of glucose (1:4) no such effect could be observed. This behavior of acetoacetate has also been confirmed in this laboratory(14). Details to be reported later.

Summary. 1. Hyperglycemia caused by daily repeated injection of acetoacetate to rabbits was completely prevented by supplementing 2% methionine to sulfhydryl deficient diet. 2. Methionine in lower concentration (0.2 to 0.5%), however, failed to prevent acetoacetate induced hyperglycemia. 3. A partial prevention of such hyperglycemia is observed when the concentration of methionine is maintained at 1% level. 4. Animals maintained on the diet supplemented with casein (26%) show normal blood sugar value up to 40 days. But as the injection is continued for another 30 days at the following doses of acetoacetate injection: 31st to 50th

day, 150 mg/kg; 51st to 70th day, 225 mg/kg, there is a slight tendency of hyperglycemia in these animals. 5. An inverse relationship between the blood glutathione concentration and blood sugar value has been observed. More the depletion of blood GSH, more is the rise of blood sugar brought about by the ketone bodies. 6. Deficiency of SH compounds seems to be an essential factor for acetoacetate induced diabetes in experimental animals.

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HYPERSENSITIVITY TO MILK AND SUDDEN DEATH IN INFANCY

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IN a review of the possible causes of sudden death in infancy, Barrett (1954) suggested that of such cases "perhaps the majority are due to the inhalation of regurgitated food into the lungs". The inhaled food might not cause death by directly obstructing the air-passages: an amount of milk too small to cause immediate asphyxia might excite an inflammatory reaction accompanied by pulmonary œdema, or the absorption of undue amounts of milk derivatives into the blood-stream might cause a state of shock.

Such effects of inhaled food or vomit, if they occur, might be due either to an inherent toxicity of the inhaled material when it enters the body by this abnormal route, or to an anaphylactic type of reaction resulting from antigenic sensitisation.

We present some experimental evidence which suggests that the second, but not the first, of these two mechanisms is indeed a possible cause of sudden death in infancy. The hypothesis is that some infants may be in a hypersensitive state from the development of antibodies to the proteins of milk absorbed from the alimentary tract. Infants often regurgitate stomach contents while asleep, and should some of this material be aspirated into the respiratory tract of an infant which has become hypersensitive, the result might be fatal.

There is no doubt that most infants fed on cow's milk develop antibodies to the proteins of the milk. Gunther, Aschaffenburg, Matthews, Parish, and Coombs (1960) using a sensitive technique—the coated tanned red-cell test of Boyden (1951)—showed milk antibodies in normal children aged 7–97 weeks in a titre up to 1000 with a modal distribution around a titre of 64.

There thus seems to be ample evidence that the majority of infants are immunised to some degree or other to the proteins of cow's milk, and the possibility exists that these or other types of antibody may sensitise the tissues of the infants. Anderson and Schloss (1923) showed that in infants with some types of nutritional disorders, milk proteins were absorbed via the alimentary tract and that subsequently precipitins to milk protein could be shown in the infants' sera, and these antibodies could sensitise guineapigs passively to anaphylaxis. According to Clein (1958), up to 6% of infants during the first few months of their lives are allergic to cow's milk.

Parish, Barrett, and Coombs (1960) showed that administration of milk over the glottis into the larynx of unsensitised conscious guineapigs was for all intents and purposes without clinical effect. On the other hand, a small amount of milk dropped into the larynx of sensitised guineapigs resulted in a characteristic anaphylactic reaction often leading to death. The reaction itself and the pathological findings in such animals were those of frank anaphylaxis and did not resemble those seen in human cot-death cases. If, however, the guineapigs were lightly anæsthetised—an experimental condition used to simulate the condition of the sleeping child—milk introduced into the larynx had quite a different effect. In the unsensitised guineapigs there was no untoward effect, and even in the sensitised animals there was a complete absence of the usual features of anaphylaxis seen in a conscious animal. Very soon after introducing the milk preparations into the larynx of an anæsthetised sensitised guineapig, the animal stopped breathing without any signs of struggle. Death sometimes occurred immediately, but the majority of animals took deep breaths at 10–30-second intervals for a variable period. Death was preceded by a short period of more rapid breathing, each respiratory effort being shallower than the preceding one, until, with a final nose-twitching, the animal died. Others lingered for 20–60 minutes before death took place, or normal respiration was gradually resumed. This sequence of events became recognised as characteristic, and was reproducible on challenge of all the anæsthetised sensitised animals. The pathological findings in the animals which died resembled those in human cot-death cases.

In the present paper we have tried to relate our experimental results more directly to the condition in man. (1) Sera from actual cases of cot death have been examined for their level of milk antibodies, as shown by the coated tanned red-cell test, in order to see if the distribution of levels is higher, as would be expected, than in randomly selected infants of comparable age. (2) To test the hypothesis that the aspirated milk proteins originate as a regurgitation from the stomach, experiments have been performed on anæsthetised sensitised guineapigs in which the stomach contents recovered from cases of cot death were introduced into the respiratory tract. Only stomach contents from infants known to have been bottle-fed at the time of death were used. (3) Experiments have been performed to see if the individual milk proteins can produce a lethal effect. (4) The pathological findings in guineapigs killed in this way are compared with those in cases of human cot death.

Materials and Methods

These have been described in detail in two previous papers (Parish et al. 1960, Gunther et al. 1960).

Milk and Milk Proteins

As National dried milk powder is the product given to the majority of bottle-fed infants, it was employed as the source of milk proteins in our previous studies. In the present work this product was again used as the sensitising antigen and the soluble proteins of the reconstituted powder used to coat the tannic-acid-treated red cells in the serological tests for milk antibodies.

The isolated proteins of cow's milk—namely, casein, α-lactalbumin, and β-lactoglobulin—were prepared as described by Gunther et al. (1960) and were kindly supplied by Dr. R. Aschaffenburg.

Testing Sera for Antibodies

The method was basically the coated tanned red-cell technique of Boyden modified as described by Gunther et al. (1960). The tanned red-cells were coated by exposing them to a 0·5% solution of protein antigen.

Sensitisation of Guineapigs and Challenge

The guineapigs each received one intraperitoneal injection

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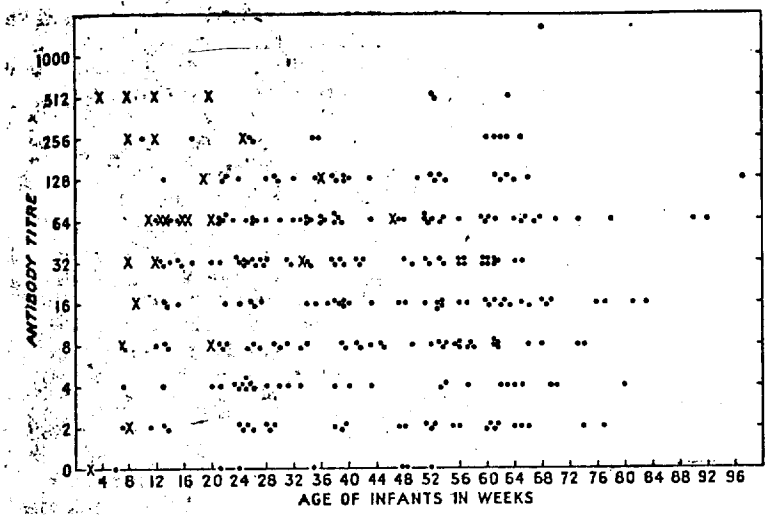

Fig. 1—Titres of serum-antibody to cow's milk proteins at various ages in 24 "cot-death" cases (X) and in 286 normal infants (●).
A strict comparison of the two categories is not possible for the reasons stated in the text.

of 1 ml. reconstituted milk powder 2% w/v. The inoculum consisted of the soluble and insoluble material.

In experiments with human stomach-contents the animals were challenged on the 18th day after injection. In experiments with milk proteins they were challenged on the 69th day.

Anæsthesia

Pentobarbitone ('Nembutal') was used as the anæsthetic in a dose of 0·01 ml. per 20 g. body-weight given intraperitoneally. The depth of anæsthesia was such that the corneal reflex was very weak or just absent and the pedal reflex still present. This was usually achieved in 15 minutes after injecting the pentobarbitone.

Challenge via the respiratory tract.—This was designed to imitate, as closely as possible, the accidental inhalation of regurgitated stomach contents by the sleeping infant. The method has been fully described and illustrated by Parish et al. (1960). With the aid of a gag and an illuminated nasal speculum, the mouth was opened and the entrance to the pharynx exposed. While the tongue was depressed by a thin metal spatula, the milk or other challenging antigen was introduced over the glottis from a specially shaped blunt needle attached to a syringe. When the animal inspired through the mouth it was possible to see the epiglottis which was the guide to the time of injection. With practice it was possible to deposit the milk drop by drop over the glottis so that it was inhaled even when the larynx was invisible to the operator. This procedure caused the minimum amount of damage to the respiratory tract.

Experimental Study

Serum Antibody Levels

Using coated tanned red cells Gunther et al. (1960) tested the sera of 286 normal children aged 7–97 weeks (fig. 1). Nearly all the sera contained measurable antibody to cow's milk proteins. In distribution the mode fell around a titre of 64. Approximately 4% had a titre of 256, 1% a titre of 512, and one serum had a titre of 1000. The plot of serum titre against the age in weeks of the child is shown in fig. 1.

We have now tested sera from 24 cases of cot death for the titre of antibodies to cow's milk. Only those cases of sudden death were included which, on the pathologist's report, could be considered as "cot-death" cases. These infants were known to have been bottle-fed at the time of death, and milk was found in their stomachs at post mortem.

The serum titres of antibody to cow's milk in the cot-death cases are also shown in fig. 1 where the distribution can be seen in relation to that found in normal infants.

The number of cases available so far is not sufficient for a final assessment, nor is the mode of selection in the two categories appropriate for a strict comparison between the serum levels in normal infants and in the cot-death cases. In the first place approximately 32% of the normal infants were known to be mainly breast-fed at 6 weeks of age, while the cot-death cases studied were all bottle-fed at the time of death. Moreover, the blood from normal infants was collected under ideal conditions and the sera frozen to −15°C within 5 hours of bleeding, while the blood from cot-death cases was obtained post mortem and often had considerable precipitates which had to be centrifuged out before testing. However the high titres found in the sera of the majority of cot-death cases are in keeping with the possibility that, had these infants aspirated milk into their respiratory tracts, a lethal hypersensitivity reaction, as seen experimentally in guinea-pigs, might have occurred.

We are not suggesting that the level of serum antibody is necessarily a measure of the degree of sensitivity, although for the type of anaphylactic reaction envisaged this cannot be excluded. It does indicate, however, that the infants have received a considerable antigenic stimulation with cow's milk and are, for this reason, potentially sensitised to these proteins.

Effect of Stomach Contents on Sensitised Guinea-pigs

In our previous experiments (Parish et al. 1960) National dried milk was used as the material for challenge. In the present paper we report a similar experiment but the material used was stomach contents from actual cot-death cases in which the infant's last feed had been from the bottle. This was used because, in the hypothesis we put forward, we believe regurgitated stomach contents are the source of the aspirated material initiating the lethal reaction.

In this experiment both sensitised and unsensitised (control) guinea-pigs were lightly anæsthetised with an intraperitoneal injection of pentobarbitone. Approximately 15 minutes later, when the desired state of anæsthesia had been reached, 0·25 ml. of a 1/2 dilution of stomach contents (0·125 ml. of the original contents) was introduced into the larynx. Stomach contents from 4 cases of cot death were used. The tenaciousness of this material necessitated its dilution with an equal volume of saline to avoid mechanical obstruction of the respiratory tract of the guinea-pig, which is, of course, much narrower than that of the human infant. The stomach contents were obtained from dead infants aged 2, 4, 8, and 22 weeks. The larger lumps found in the stomach of the oldest child were first broken down in a Griffith grinder.

TABLE I—CLINICAL EFFECTS OF INHALING STOMACH CONTENTS FROM HUMAN COT-DEATH CASES BY NORMAL AND BY PREVIOUSLY SENSITISED GUINEA-PIGS LIGHTLY ANÆSTHETISED TO SIMULATE SLEEP

Challenge with stomach contents of dead infant	Dose of diluted stomach contents	Sensitised guinea-pigs		Unsensitised guinea-pigs	
		Reactions	Deaths	Reactions	Deaths
A, aged 2 weeks	0·25 ml.	D D D D + + +	4/5	+ − − − −	0/5
B, aged 4 weeks	0·25 ml.	D D D + + −	3/5	− − − − −	0/5
C, aged 8 weeks	0·25 ml.	D D D D	4/4	− − − −	0/4
D, aged 22 weeks	0·25 ml.	D D D D + +	4/5	+ + − − −	0/5

Scale of severity of clinical effects:
D = death.
+ + + = severe respiratory embarrassment just insufficient to be fatal.
+ + = definite clinical disturbance but not severe.
+ = one or two signs of respiratory embarrassment but always mild.
− = no response.

In the unsensitised guineapigs no deaths, and in the great majority of cases no effects, were observed. On the other hand most of the sensitised animals exhibited the syndrome already described and died silently, without any struggle, and in a very short time. Three animals recovered after a protracted period during which there were long spells of apnœa interrupted only by occasional weak gasps. One of the sensitised animals was apparently unaffected. The results are summarised in table I.

Effect of Milk Proteins on Sensitised Guineapigs

In our earlier report (Parish et al. 1960) we recorded the level of milk antibodies and the clinical sensitivity to challenge with milk at 17, 38, 64, and 92 days after a single sensitising injection. Although in some animals the level of serum-antibody appeared to fall with the passage of time the animals were as sensitive clinically to the aspiration of milk on the 92nd day as they were earlier. We wished to see if a similar lethal reaction could be produced by the individual component proteins of cow's milk.

The guineapigs were sensitised as before by injecting intraperitoneally 2% w/v reconstituted National dried milk in a dose of 1 ml. On the 69th day they were challenged via the respiratory tract while under light anæsthesia with 0·25 ml. of: (*a*) the soluble proteins in 10% w/v reconstituted milk powder (approximately 1% soluble proteins), (*b*) 1% casein, (*c*) 1% α-lactalbumin, or (*d*) 1% β-lactoglobulin. The day before challenge each animal was bled and the serum subsequently tested for antibodies to reconstituted dried milk, and to the component protein with which the guineapig was challenged.

The results of the serological tests, and the clinical effects on challenge are recorded in table II. Casein, as might be expected, proved to be as effective as whole milk in producing lethal reactions. In one of the previous papers (Gunther et al. 1960) similarly sensitised guineapigs tested 17 days after injection had antibodies to α-lactalbumin but none to β-lactoglobulin. In the present experiment no antibodies could be shown to α-lactalbumin 69 days after injection. Clinically only very mild reactions were produced on challenging with α-lactalbumin, but with β-lactoglobulin the reactions were as severe as with casein or whole milk.

TABLE II—LEVEL OF SERUM ANTIBODY TO MILK PROTEINS ON 69TH DAY AFTER INJECTION AND CHALLENGE OF SENSITISED GUINEAPIGS WITH COMPONENT PROTEINS OF COW'S MILK

Guinea-pig	Challenged with	Serum antibody titre to				Clinical effect on challenge‡
		Reconst. National dried milk	Casein	α-lact-albumin	β-lacto-globulin	
1	Soluble material in reconstituted National dried milk*	16	..	..	..	++
2		16	..	..	..	D
3		32	..	..	..	+++
4		16	..	..	..	D
5		..	..	..	..	D
6	Casein†	8	16	..	..	D
7		16	16	..	..	++
8		128	64	..	..	D
9		16	16	..	..	D
10		16	16	..	..	D
11	α-lactalbumin†	8	..	0	..	+
12		32	..	0	..	+
13		32	..	0	..	+
14		8	..	0	..	w
15		16	..	0	..	−
16	β-lactoglobulin†	16	..	..	0	D
17		8	..	..	0	++
18		4	..	..	0	D
19		64	..	..	32	D
20		64	..	..	4	D

0 indicates a titre <2.
Scale of severity of clinical effects—see table I.
* Made up 10% w/v—approximately 1% soluble protein.
† A 1% solution.
‡ Of thirty unsensitised (control) anæsthetised guineapigs challenged with soluble or insoluble milk preparations (Parish et al. 1960) no reaction was observed in twenty-six—one showed a very mild reaction and two a moderate reaction. One animal died, but on subsequent investigation its serum was shown to contain what appeared to be a naturally occurring antibody to cow's milk proteins to a titre of 16.

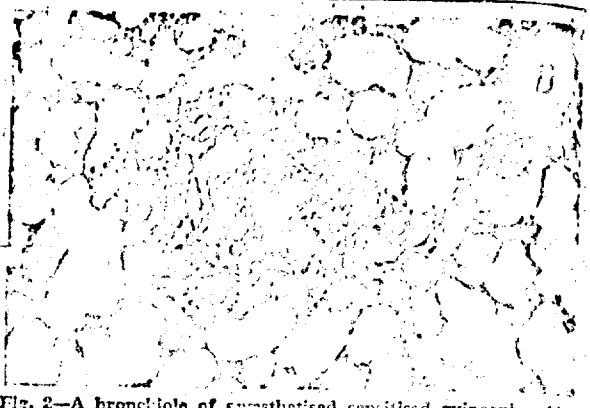

Fig. 2—A bronchiole of anæsthetised sensitised guineapig which died soon after the introduction into its trachea of 0·25 ml. of diluted (50%) stomach contents from a case of cot death. (Hæmatoxylin and eosin. ×120.)

The bronchiole is so full of desquamated epithelial cells that in the photograph it is scarcely recognisable.

We have thus shown that lethal reactions can be produced with casein and β-lactoglobulin. On the other hand only mild reactions were obtained with α-lactalbumin. However, further guineapigs will have to be tested at different periods after sensitisation before this protein can be considered less noxious than the other two.

Pathological Findings in Sensitised Guineapigs

The histopathology of the lungs of sensitised guineapigs which died after inhalation, when anæsthetised, of total reconstituted dried milk, or its soluble or insoluble fractions, has already been described (Parish et al. 1960). All the changes observed in the lungs of similarly sensitised guineapigs which died after the inhalation of casein or β-lactoglobulin closely resembled those in the earlier experiments. In those sensitised animals challenged with β-lactoglobulin or casein, the chief features were generalised congestion with occasional areas of partial collapse, and serous exudate or œdematous transudate. Cellular infiltration in the œdematous areas, or in the peribronchial, or periarteriolar connective tissue consisted chiefly of mononuclear cells. Many of these were macrophages with variable numbers of lymphocytes and, in some animals, plasma-cells; but there were few polymorphonuclear cells. A common finding was the desquamation of large numbers of single, intact, apparently normal, epithelial cells of the bronchial wall into the lumen of the small bronchioles.

The histopathological changes in the lungs of sensitised anæsthetised guineapigs which had died as a result of inhaling small amounts of the stomach contents obtained from cot-death cases were similar to those observed in sensitised animals inhaling milk. Generalised congestion was usual, but there was only an occasional small area of œdema and little cellular infiltration. The infiltrating cells were chiefly macrophages, though there was an increased number of polymorphonuclear leucocytes—more than observed in animals challenged by milk. The majority of the leucocytes were eosinophils, and in some preparations they were found also in the peribronchial connective tissue. There was some evidence that the bronchioles and arterioles had undergone contraction, but further study of this feature is required. Many of the medium-sized and smaller bronchioles contained large numbers of desquamated cells; sometimes these completely filled the lumen (fig. 2). Bronchioles thus affected could be found in all the lung material, but the number varied from animal to animal. The trachea was usually congested and in some cases infiltrated by eosinophils; the epithelium of the trachea was not desquamated.

The unsensitised—i.e., normal anæsthetised control guineapigs which had inhaled the stomach contents obtained from the same human subjects, and subsequently killed by an

overdose of pentobarbitone—had different histopathological changes in their lungs. There were large areas of partial collapse and œdema, or serous exudate, infiltrated with moderate numbers of macrophages and polymorphs of both the heterophil and eosinophil type. All areas of the lung were congested and there was an occasional small hæmorrhage into the alveoli. The epithelium of the majority of the bronchioles was intact; if it was not, the desquamation was in the form of sheets or strips of epithelium, and for the most part the individual cells were not detached from one another (fig. 3).

The differences in degree of œdema and cellular infiltration between the unsensitised and sensitised guineapigs challenged with human stomach contents probably depend on the time that the irritant material remained in the living animal. The majority of the sensitised animals died within 5 minutes, and all within 10 minutes, whereas the unsensitised animals were killed about 3 hours after challenge. Previous experiments on normal animals revealed that the mode of killing by pentobarbitone did not cause changes of this type.

The histopathological findings in sensitised guineapigs, dying after inhalation of milk proteins, are very similar to those described and illustrated in cot death by Barrett

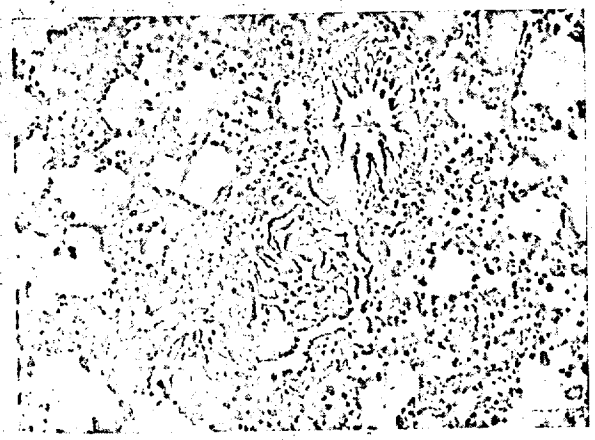

Fig. 3—Bronchioles of an unsensitised guineapig which was killed 3 hours after receiving the same dose of the same stomach contents as fig. 2. (H. & E. ×120.)

The introduction of the stomach contents was without clinical effect. The upper bronchiole is normal; the bronchiolar epithelium of guineapigs is normally more folded than that of man. In the lower bronchiole, the lumen is occupied by strips of detached epithelium, but the appearance is different from that in fig. 2.

(1954) and Stewart (1957). The lungs of cot-death subjects are usually congested, and the engorgement of the capillaries is often accompanied by œdema, small hæmorrhages, foci of partial collapse, and an increase in the number of macrophages in the alveoli. The walls of the bronchi are infiltrated by lymphocytes and plasma cells; sometimes there are also smaller numbers of polymorphonuclear leucocytes. Desquamation of the bronchial epithelium is commonly seen (figs. 4 and 5) and is often of the type described by Bodian and Heslop (1956), who thought that the presence within the bronchi of large numbers of individual desquamated cells, embedded in mucus was characteristic of cot death, although it did not occur in every case.

All of these histopathological features of cot death were also seen in the sensitised anæsthetised guineapigs which died after inhalation of small amounts of milk, or of stomach-contents containing milk. Perhaps the most significant common finding was the presence of large

Fig. 4—Bronchiole from case of cot death, showing lumen filled with desquamated epithelial cells. (H. & E. ×100.)

numbers of individual intact epithelial cells in the lumina of the bronchioles. The appearance was the same as that described by Bodian and Heslop (1956) except that in the guineapigs there was little if any mucus. This difference may be due to an anatomical difference between man and guineapig, for there are very few mucous glands in the trachea and bronchi of the guineapig, and few goblet-cells in the epithelium. Another difference between the human and the animal material is that in the guineapigs no desquamation of epithelium was observed in the trachea, though it occasionally occurred in the large bronchi. Desquamation of epithelium is, however, a common postmortem change, which would be expected to be greater in the human than in the animal material because of the unavoidably longer interval between death and necropsy.

Discussion

Sudden death of infants is by no means uncommon, and the Registrar General's data showed that in England and Wales in 1955, there were 1432 such deaths accounting for 20·5% of the 6993 deaths between the ages of 2 weeks and 2 years (Banks 1958).

The usual history is that of a baby dying in its sleep. As a rule the babies are between 1 month and 6 months of age, though the incidence appears to be highest at about 3 months. These deaths are attributed by many to a fulminating infection, although attempts to demonstrate a causal organism, either bacteria or virus, have been unsuccessful in most cases (Banks 1958).

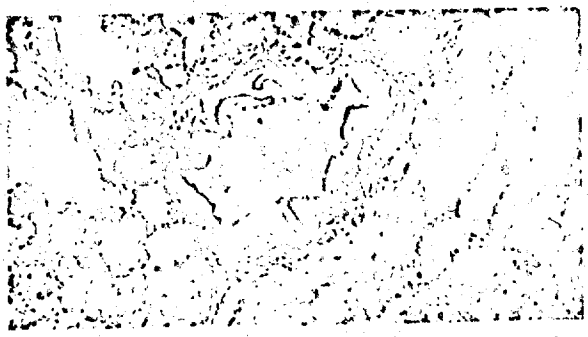

Fig. 5—Bronchiole of infant believed to have died of suffocation. (H. & E. ×100.)

The epithelium is intact except at the top left, where it has become partially detached. Such detachment often occurs post mortem.

The most common foreign proteins to which a baby is exposed are those of cow's milk, either as fresh milk, or as a preserved-milk product, which is also a constituent of proprietary preparations for infants. It has previously been shown that babies may be sensitised to milk products (Anderson and Schloss 1923, Tallerman 1934) and this sensitivity may cause disease on contact with the milk antigen (Clein 1958). In the preliminary experiments, where examination was made of the sera of normal babies between 7 and 97 weeks of age for agglutinating antibodies to cow's milk, the large majority were found to have been "immunised" to some degree or other, and their tissues were probably sensitised by some form of antibody as a result of this immunisation (Gunther et al. 1960). When, as in fig. 1, the antibody titres to milk in the serum of babies believed to be typical cases of cot death are superimposed on those obtained from normal infants, it can be seen that in any age group the titres of the cot-death cases tend to be high when compared with the normal, indicating that a considerable degree of sensitivity could be present.

In experiments performed on guineapigs (Parish et al. 1960), which have been confirmed by the data presented in this paper, it was found that sensitised guineapigs in an anæsthetised state died after inhalation of milk proteins. Death was not typical anaphylactic shock as observed in the conscious state, but the animal, without any struggle, stopped breathing either at once or after a brief episode of irregular respiration. Animals not sensitised to milk could inhale up to 1·0 ml. without significant effect. The histopathological changes following challenge of the sensitised animals had much in common with those described in cases of cot death in infants.

It has thus been demonstrated that sensitised animals, when unconscious, can be killed quickly, silently, and without trace of struggle by the inhalation of whole milk, or, at least, by two constituent proteins of milk, casein and β-lactoglobulin.

It is well known that babies frequently regurgitate small quantities of food during sleep and often leave traces on the pillow. In our hypothesis it is suggested that inhalation of regurgitated material can induce a lethal reaction, and that only small amounts need be aspirated. The quantity aspirated by the infant may be of the order of 1·0 ml., on a comparison of the weights of the test guineapigs and cot-death subjects.

We have been able to show that many babies have some antibody to cow's milk, and that guineapigs with a similar serum-antibody level can be killed quietly by aspiration of small quantities of milk.

If the challenge agent in babies were food material from the stomach regurgitated during sleep, it had to be shown that this material retained its antigenic properties sufficiently to cause shock on contact with tissues sensitised to milk. This was found to be so. The stomach contents taken from 4 cases of cot death in which the infants were known to be bottle-fed, when inhaled in small amounts by lightly anæsthetised guineapigs sensitised to milk proteins, resulted in death, quietly and without struggle, in 15 of the 19 challenged animals. The histopathological features were similar to those observed in cot death of infants. An equal number of anæsthetised guineapigs, not sensitised to milk proteins, which had inhaled the same stomach-content material displayed no effects, or scarcely appreciable respiratory embarrassment, and there were no deaths.

These experiments provide a model of a possible cause of cot death in infants. If the serum level of antibody is a valid indication, the majority of babies are sensitised to some degree to cow's milk, and it is possible that a normal healthy baby may be put to bed, and, during its sleep regurgitate and inhale sufficient material to cause an antigen-antibody reaction in the sensitised tissue of the lungs, causing physiological and pathological changes resulting in the death of the infant.

Further investigation of this problem is being undertaken. An attempt is being made to prove that an antigen-antibody reaction has taken place in the lungs of such infants and that this reaction is of sufficient magnitude to cause death. The nature or the degree of the sensitivity may be an important factor, for the number of babies that have a substantial titre of circulating antibodies to milk is large compared to the small number of cot-death cases, though another limiting factor may be the frequency with which aspiration occurs. Nevertheless, the fact that babies do become sensitised to cow's milk proteins, and that inhalation of this material could conceivably be the cause of cot death in a young infant, should be another inducement to breast-feed young babies where practicable.

Summary

Experimental evidence is presented to support the hypothesis that cot death in infants may be due to an anaphylactic type of reaction consequent on the inhalation of cow's milk proteins regurgitated from the stomach during sleep.

Serological tests show that most infants fed on cow's milk develop antibodies to milk proteins.

Experiments on anæsthetised guineapigs, sensitised to the proteins of cow's milk, show that many such animals die rapidly and without struggling when a small quantity (0·25 ml.) of: (*a*) cow's milk, (*b*) stomach contents from cases of "cot death", (*c*) a 1% solution of casein, or (*d*) a 1% solution of β-lactoglobulin is introduced into the air passages.

The histopathological changes in the lungs of the experimental animals resemble those found in cases of cot death.

We wish to acknowledge the helpful discussion with the members of the steering committee and the scientific subcommittee of the "Enquiry into sudden death in infancy" under the respective chairmanships of Prof. Leslie Banks and Sir Samuel Bedson and to thank this body and the Nuffield Foundation for a grant to cover the cost of animals used in these experiments. They are most grateful to Dr. H. R. M. Johnson for his help in the collection of material from cot-death cases, and to Dr. N. R. Butler and the pædiatric staff, University College Hospital, for their help in obtaining serum-samples.

We thank Miss Brenda Disbrey and her staff for the painstaking work in the preparation of the histological material, Mr. B. W. Gurner for drawing fig. 1, and Mr. S. W. Patman for taking the photographs.

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STUDIES ON THE ALLERGENICITY OF COW'S MILK

I. The Allergenic Properties of Alpha-casein, Beta-lactoglobulin and Alpha-lactalbumin

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TWENTY years ago, studies on the anaphylactogenic properties of milk[1] were carried out with relatively crude protein fractions. The results obtained were of only limited value, since the lactalbumin fraction contained considerable globulin and there were both lactalbumin and lactoglobulin in the casein fraction.

We have now obtained highly purified protein fractions of milk, as tested by electrophoresis. The object of this study is to determine the allergenicity and allergenic purity of these proteins in milk.

MATERIALS

The protein fractions of bovine (cow's) milk under study are alpha-casein, beta-lactoglobulin and alpha-lactalbumin, which constitute approximately 60%, 11% and 2.4%, respectively, of the total proteins of milk.[2]

Preparation of Pure Proteins of Milk

The fractions of protein shall be designated as the McMeekin-Gordon milk proteins.*

Unpasteurized, mixed, commercial, skimmed milk was used for the preparation of the milk proteins.

Casein was prepared by the method described by Hipp, Groves and McMeekin.[3] This casein fraction contains alpha-casein, beta-casein and gamma-casein.

Alpha-casein was isolated and crystallized by the method of Hipp, Groves and McMeekin.[4]

Beta-lactoglobulin was isolated and crystallized by the method of Palmer,[5] modified by using ammonium sulfate instead of sodium sulfate for precipitation of the protein.

Alpha-albumin was isolated and crystallized by the method of Gordon and Zeigler.[6]

These protein fractions were all shown to be highly purified by electrophoretic analysis.

The final solutions to be tested were prepared* by suspending the protein fractions as a 5.0% extract in buffered saline and adjusting to pH 7.8 by the addition of 0.1 normal solution of sodium hydroxide. After 24 hours, the resulting clear solutions were passed through a Seitz filter. The total nitrogen contents of the final solutions were alpha-casein, 6.35 mg/ml; beta-lactoglobulin, 6.13 mg/ml; alpha-lactalbumin, 6.4 mg/ml.

METHODS

Guinea pigs weighing 200 to 250 gm were sensitized and challenged with the purified protein fractions as tabulated (Table I). The diet had previously contained no milk proteins.

All sensitizing injections were given intraperitoneally and/or subcutaneously. All challenging injections into intact animals were given intravenously into the jugular vein.

Anaphylaxis experiments with isolated ileum[7,8] were carried out by removing the ileums from sensitized guinea pigs and suspending them in a bath containing 5.0 ml of freshly made, oxygenated Tyrode solution

* They were prepared by Dr. T. L. McMeekin and Dr. W. G. Gordon, Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia, Pennsylvania.

* Center Laboratories, Inc., Port Washington, N.Y.

* Posthumously submitted.
(Accepted April 8, 1958; submitted February 19.)
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TABLE 1*

A. Allergenic Superiority of Beta-lactoglobulin as Compared with Alpha-lactalbumin and Alpha-casein

Number of Animals	*Single Sensitizing Injection, 1.0 mg Sc*	*Challenge 3 Weeks Later, 0.1 ml PSM, Iv*	*Result*
10	Alpha-lactalbumin	All negative	
10	Alpha-casein	All negative	
10	Beta-lactoglobulin	7	++++
		1	++
		1	+
		1	0

B. Enhancement of Allergenicity of Alpha-lactalbumin and Alpha-casein by Sensitization with Multiple Doses

Number of Animals	*Multiple Sensitizing Injections; 1 mg Sc+1 mg Ip; 2 Days Later 1 mg Sc*	*Challenge 3 Weeks Later, 0.1 ml PSM, Iv*	*Result*
10	Alpha-lactalbumin	4	++++
		1	+++
		2	++
		1	+
		2	0
10	Alpha-casein	3	++++
		2	++
		4	-
		1	0
10	Beta-lactoglobulin	8	++++
		1	+
		1	0

* Abbreviations in Table:
Sc = subcutaneously; Iv = intravenously; Ip = intraperitoneally.
PSM = pasteurized skimmed milk.
++++ = Anaphylactic death—dyspnea, convulsions, collapse, apnea and death.
+++ = Severe anaphylaxis—dyspnea, convulsions, collapse and recovery.
++ = Moderate anaphylaxis—dyspnea, convulsive movements, moderate collapse, recovery.
+ = Mild anaphylaxis—dyspnea and scratching.
0 = No reaction.

maintained at 37.5°C. All contractile reactions were registered on a smoked drum, rotating at a speed of 2.5 cm/sec. Histamine was used to test the viability and contractility of each segment of ileum at the beginning and at the end of each experiment.

After an anaphylactic contraction was obtained with a specific antigen, the segment of ileum was allowed to relax completely and then the bath was washed out three times with fresh Tyrode solution. The segment was again tested with the same antigen to demonstrate specific desensitization, and the bath again rinsed three times with Tyrode solution. This procedure was then carried out with each challenge with subsequent heterologous antigenic contacts.

RESULTS

Allergenic Specificity of Bovine Crystalline Beta-lactoglobulin, Alpha-lactalbumin and Alpha-casein

Ten guinea pigs were sensitized with two injections of 1.0 mg each of beta-lactoglobulin given subcutaneously 2 days apart. Three weeks later they were each challenged in turn with intravenous injections of 0.1 mg first of alpha-lactalbumin, then of alpha-casein and, finally, of the homologous beta-lactoglobulin. None reacted to the two heterologous protein fractions, while all died in anaphylaxis after challenge with the homologous beta-lactoglobulin. These results demonstrate a high degree of sensitization to the beta-lactoglobulin with no evidence of cross reaction with alpha-lactalbumin or alpha-casein fractions, thus confirming the purity of the beta-lactoglobulin.

In order to rule out the possibility of primary toxicity of beta-lactoglobulin, five normal animals were given a single intravenous injection of 0.1 mg of this fraction. None showed any reaction.

Sixteen animals were sensitized with two injections of 1.0 mg each of alpha-lactalbumin, given subcutaneously 2 days apart. Three weeks later they were challenged in turn with intravenous injections of 0.1 mg of each of the two heterologous fractions, first beta-lactoglobulin and then alpha-casein, to which they showed no reactions.

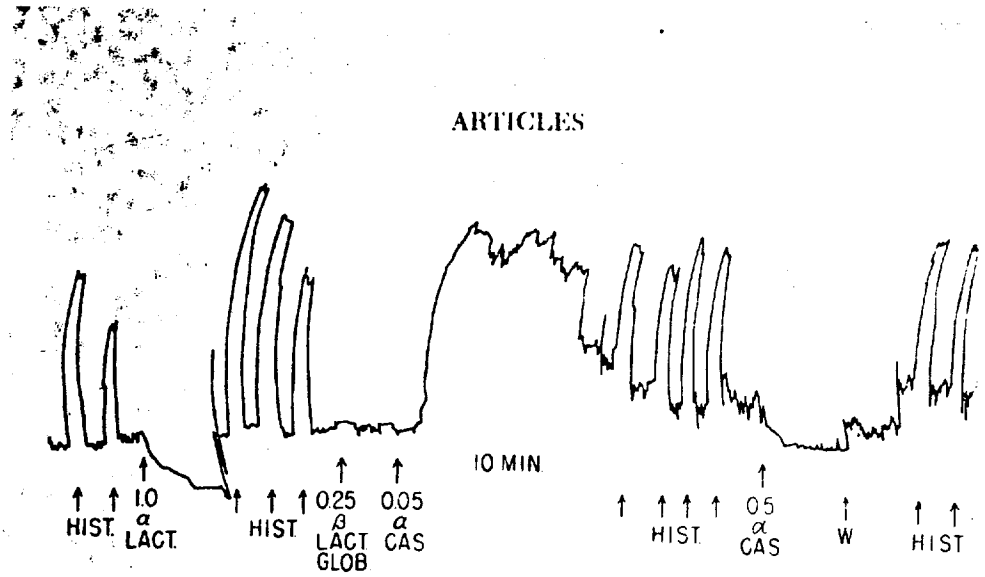

FIG. 1. Evidence of antigenic purity of alpha-casein fraction. Tracing of contractions of segment of guinea pig ileum sensitized to alpha-casein. 6.0 ml bath. HIST. = histamine followed by 3 washes. Three washes before each new addition of material. W = 3 washes. From left to right: two normal histamine contractions; no reaction to alpha-lactalbumin; three histamine contractions; no reaction to beta-lactoglobulin; specific anaphylactic contraction to alpha-casein; four histamine contractions; no contraction with alpha-casein (desensitization); two normal histamine contractions.

Finally they were challenged with the homologous alpha-lactalbumin, after which eight animals had anaphylactic reactions: three fatal and five mild to moderate in degree; eight had no reactions. These results demonstrated a moderate degree of allergenicity of alpha-lactalbumin, of a lower order than that of beta-lactoglobulin, and no evidence of cross reactions with the heterologous fractions.

Experiments with the isolated ileum of the guinea pig fortified the evidence derived from anaphylaxis in intact animals, and showed that alpha-casein is also allergenically pure. It was demonstrated that the isolated segment of ileum from an animal sensitized to alpha-casein reacts to neither of the heterologous fractions (Fig. 1).

Relative Allergenicity of Beta-lactoglobulin, Alpha-lactalbumin and Alpha-casein

In preliminary experiments it was found that, while a single subcutaneous injection of 1.0 mg of alpha-lactalbumin or alpha-casein was an inadequate sensitizing dose, two or three such injections, given several days apart, gave a high degree of sensitization. In contrast, a single injection of 1.0 mg of beta-lactoglobulin resulted in a high degree of sensitization, for 7 of 10 animals died of anaphylactic shock after challenge with pasteurized skimmed milk (Table I, A).

When animals were sensitized with multiple injections, a definite sensitivity was established to the alpha-lactalbumin and alpha-casein (Table I, B), while multiple sensitizations with beta-lactoglobulin did not increase the high degree of sensitivity established after a single injection of that substance.

From these data it is seen that of the protein fractions of milk studied, beta-lactoglobulin is the most allergenic, while alpha-lactalbumin and alpha-casein have appreciably lower allergenicity. However, with multiple sensitizing injections, the latter two fractions are capable of sensitizing animals.

SUMMARY

It seems apparent from the present study that each of the McMeekin-Gordon protein fractions of milk is immunologically homogeneous.

Of the three fractions, beta-lactoglobulin is the most allergenic, while alpha-lactalbumin and alpha-casein are of a much lower order of allergenicity.

While sensitization by parenteral injec-

tion is artificial and may not thus apply strictly to natural sensitization to milk through ingestion, the data indicate that lactoglobulin is the dominant allergen of the three studied.

Heretofore, lactalbumin has been considered the major protein in the production of milk allergy.[9] The present studies furnish evidence against the lactalbumin fraction as the potent allergen in clinical practice and evidence in favor of the lactoglobulin fraction as the allergen responsible for the majority of cases of milk allergy.

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SUMMARIO IN INTERLINGUA

Studios Relative Al Allergenicitate De Lacte Bovin I.

Tres fractiones proteinic de lacte bovin—alpha-caseina, beta-lactoglobulina, e alpha-lactalbumino (le quales esseva obtenite in forma crystallin e, secundo lor analyse electrophoretic, altemente purificate)—esseva investigate con respecto a lor puritate allergenic, i.e. lor allergenicitate relative.

Porcos de India recipeva injectiones del mentionate fractiones proteinic, sequite per stimulation con fractiones heterologe e homologe. In le majoritate del casos, le stimulation esseva effectuate per injectiones intravenose in animales intacte, sed un certe numero de experimentos esseva executate con isolate segmentos de ileum prendite ab porcos de India sensibilisate.

Sensibilisation con le un o le altere del tres fractiones proteinic non resultava in ulle caso in un reaction anaphylactic sub le effecto de un heterologe fraction de proteina. Isto provava que le tres fractiones esseva immunologicamente pur.

Inter le tres fractiones studiate, beta-lactoglobulina se monstrava le plus allergenic, durante que alpha-lactalbumina e alpha-caseina exhibiva un ordine multo inferior de allergenicitate.

Usque nunc, lactalbumina esseva considerate como le factor le plus importante in le disveloppamento de allergia a lacte. Le presente studios ha producite provas contra le incrimination de lactalbumina como potente allergeno in le practica clinic e pro le conception que lactoglobulina es plus probabilemente responsabile pro le majoritate del casos de allergia a lacte. Tamen, viste que sensibilisation per injection parenteral es artificial e possibilemente non simula strictemente le sensibilisation natural a lacte per ingestion, un conclusion definite debe attender investigationes additional.

Pediatrics 22:642-652, 1958

STUDIES ON THE ALLERGENICITY OF COW'S MILK

II. Effect of Heat Treatment on the Allergenicity of Milk and Protein Fractions from Milk as Tested in Guinea Pigs by Parenteral Sensitization and Challenge

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THE HEATING of native proteins may result in complete or partial loss of specific immunologic properties. This denaturation is believed to be due to coagulation resulting from the reaction between the protein and moist heat.[1]

This study was designed to investigate the alterations of allergenicity of the alpha-lactalbumin, beta-lactoglobulin and alpha-casein in a heat-processed cow's milk.

MATERIALS AND METHODS

The preparation of pure proteins, alpha-lactalbumin, beta-lactoglobulin and alpha-casein, were fully described in a previous paper.[2]

Pasteurized skimmed milk used in certain of these experiments was obtained from ordinary commercial sources.

The heated milks under study will be referred to as "heat-denatured milk," liquid or dry. These are the same as the commercially available preparations* which are heated before evaporation. The liquid and dried milks are treated similarly except that the latter is flash dried after heat evaporation. In this study the milks were reconstituted with distilled water. The final product has a content of protein of 1.7 gm/100 ml, as compared to 3.5 gm/100 ml for the pasteurized skimmed milk.

Anaphylaxis tests, utilizing intact guinea pigs and isolated segments of guinea pig ileum, were employed as described in a previous paper.[2]

* Similac Powder and Similac Liquid, kindly supplied by the Ross Laboratories, Columbus, Ohio.

RESULTS

Effect of Heat Denaturation of Milk on the Sensitizing Qualities of the Various Protein Fractions of Milk

In these experiments the sensitizing dose was relatively large. Ten animals received 5.0 ml each intraperitoneally of powdered, heat-denatured milk (Table I, A). All reacted negatively when challenged with the relatively large intravenous injection of 0.3 mg of alpha-lactalbumin. When this injection was followed by a second challenge with 0.3 mg of alpha-casein intravenously, six showed fatal anaphylaxis, two, mild anaphylaxis and two, no reaction.

Ten additional animals, similarly injected, were challenged with beta-lactoglobulin. Fatal anaphylaxis was demonstrated in nine and no reaction in one (Table I, B).

Ten animals, similarly injected, but with liquid instead of powdered, heat-denatured milk, were challenged intravenously with 0.3 mg of alpha-lactalbumin, and none reacted. When they were challenged 1 hour later with 0.1 mg of beta-lactoglobulin, fatal anaphylaxis was observed in seven, mild to moderate anaphylaxis in two, and no reaction in one (Table I, C).

Effect of Heat Denaturation on the Allergenicity of Milk as Tested by Challenge of Animals Sensitized to the Protein Fractions

Each of 10 animals received three injec-

[1] Posthumously submitted.

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TABLE I*

EFFECT OF HEAT DENATURATION OF MILK ON THE SENSITIZING QUALITIES OF ALPHA-LACTALBUMIN, BETA-LACTOGLOBULIN AND ALPHA-CASEIN

	Animal	*Sensitization, 5 ml Ip*	*Challenge, 0.3 mg Iv*	*Result*	*Second Challenge, Iv*	*Result*
		Powdered HDM	Alpha-lactalbumin		Alpha-casein 0.3 mg	
	1			0		+
	2			0		++++
	3			0		+
	4			0		++++
	5			0		++++
A	6			0		++++
	7			0		++++
	8			0		0
	9			0		++++
	10			0		0
		Powdered HDM	Beta-lactoglobulin			
	11			0		
	12			++++		
	13			++++		
	14			++++		
	15			++++		
B	16			++++		
	17			++++		
	18			++++		
	19			++++		
	20			++++		
		Liquid HDM	Alpha-lactalbumin		Beta-lactoglobulin, 0.1 mg	
	21			0		++
	22			0		++++
	23			0		++++
	24			0		+
	25			0		++++
C	26			0		++++
	27			0		++++
	28			0		0
	29			0		++++
	30			0		++++

* In this and subsequent tables:
Ip = intraperitoneal; Iv = intravenous; Sc = subcutaneous.
HDM = heat denatured milk; PSM = pasteurized skimmed milk.
++++ = Anaphylactic death—dyspnea, convulsions, collapse, apnea, death.
+++ = Severe anaphylaxis—dyspnea, convulsions, collapse and recovery.
++ = Moderate anaphylaxis—dyspnea, convulsive movements, moderate collapse and recovery.
+ = Mild anaphylaxis—dyspnea and scratching.
0 = No reaction.

tions of 1.0 mg of alpha-lactalbumin (Table II, A). When they were challenged 3 weeks later with 0.1 ml of heat-denatured milk intravenously, they showed no reaction. However, on intravenous injection of the same dose of pasteurized skimmed milk 1 hour later, eight animals had anaphylactic reactions, of which four were fatal.

Each of 10 animals received multiple injections of 1.0 mg of beta-lactoglobulin (Table II, B). When challenged with heat-denatured milk intravenously 3 weeks later, three had anaphylactic reactions of mild degree while seven had no reaction. When these same animals were challenged 1 hour later with pasteurized skimmed milk, 9 of

TABLE II

EFFECT OF HEAT DENATURATION OF MILK ON ITS ALLERGENICITY, AS TESTED BY CHALLENGE OF ANIMALS SENSITIZED TO PROTEIN FRACTIONS FROM MILK

	Animal	Sensitizing Injection, 1 mg Sc+1 mg Ip; 2 Days Later, 1 mg Sc	Parenteral Challenge 3 Weeks Later, 0.1 ml Iv		
			Result of First Injection of HDM	Second: 1 hr Later	Result
A	1	Alpha-lactalbumin	0	PSM	++++
	2		0		+++
	3		0		++
	4		0		++++
	5		0		++
	6		0		++++
	7		0		+
	8		0		0
	9		0		++++
	10		0		0
B	11	Beta-lactoglobulin	0	PSM	0
	12		0		+
	13		0		++++
	14		0		++++
	15		+		++++
	16		0		++++
	17		0		++++
	18		0		++++
	19		+		++++
	20		+		++++
C	21	Alpha-casein	0	PSM	0
	22		++++		
	23		0		+
	24		0		+
	25		++++		
	26		++++		
	27		+		+
	28		+		+
	29		+		++
	30		+		++

the 10 had anaphylactic reactions, 8 of which were fatal.

Ten animals each received multiple injections of 1.0 mg of alpha-casein (Table II, C). When challenged with heat-denatured milk, seven had anaphylactic reactions, three of which were fatal. Rechallenge of the seven surviving animals with pasteurized skimmed milk 1 hour later produced mild to moderate reactions in six, two of which had previously had negative reactions, while no reaction occurred in the seventh animal.

The heat stability of alpha-casein with respect to allergenicity was confirmed by experiments in which tests for anaphylaxis were carried out with isolated segments of ileum from guinea pigs sensitized to alpha-casein. Thus, such a segment reacted negatively to alpha-lactalbumin but showed a typical anaphylactic reaction to heat-denatured milk (Fig. 1).

Finally, the heat lability with respect to allergenicity of alpha-lactalbumin was confirmed by demonstrating that an isolated segment of ileum from a guinea pig sensi-

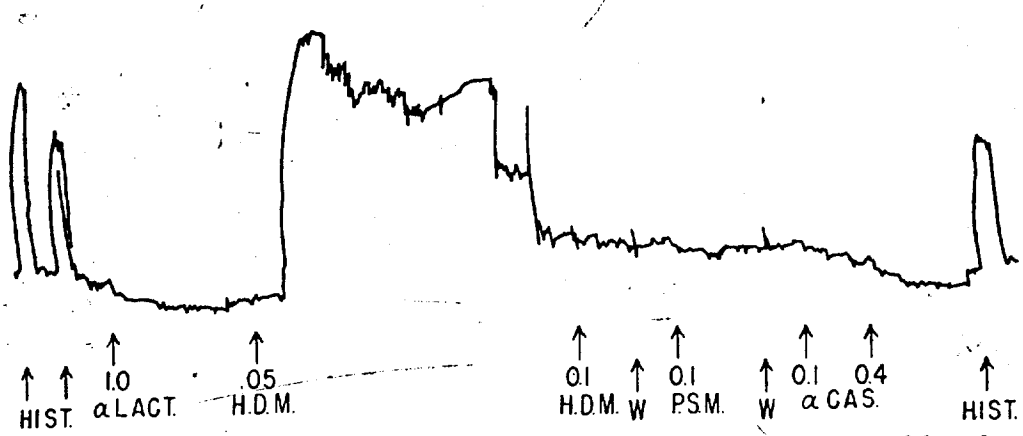

FIG. 1. In-vitro demonstration of heat stability of alpha-casein. Tracing of isolated segment of ileum from guinea pig sensitized to alpha-casein. 6.0 ml bath. From left to right: HIST. = histamine, 0.2 μg and 0.1 μg with three washes after each addition; α-LACT. = 1.0 mg alpha-lactalbumin–no response; H.D.M. = 0.05 ml heat-denatured milk–anaphylactic contraction with gradual return to base line; 0.1 ml heat-denatured milk–no reaction (desensitization); W = three washes; P.S.M. = 0.1 ml pasteurized skimmed milk–no reaction (desensitization complete to H.D.M.); W = three washes; α-CAS. = 0.1 and 0.4 mg of alpha-casein–no reaction (desensitization to α-casein); 0.1 μg histamine, showing that segment is viable at conclusion of experiment.

tized to this fraction showed no response to heat-denatured milk, but showed a typical anaphylactic reaction to pasteurized skimmed milk (Fig. 2).

COMMENT

These anaphylaxis experiments show the influence of heat on the allergenicity of milk and fractions of milk.

The allergenicity of the alpha-lactalbumin fraction was eliminated in the heat-denatured milk, whether this milk is used as a sensitizing or shocking agent.

The beta-lactoglobulin fraction, previously shown to be an extremely potent antigen, retained its sensitizing qualities in the heat-

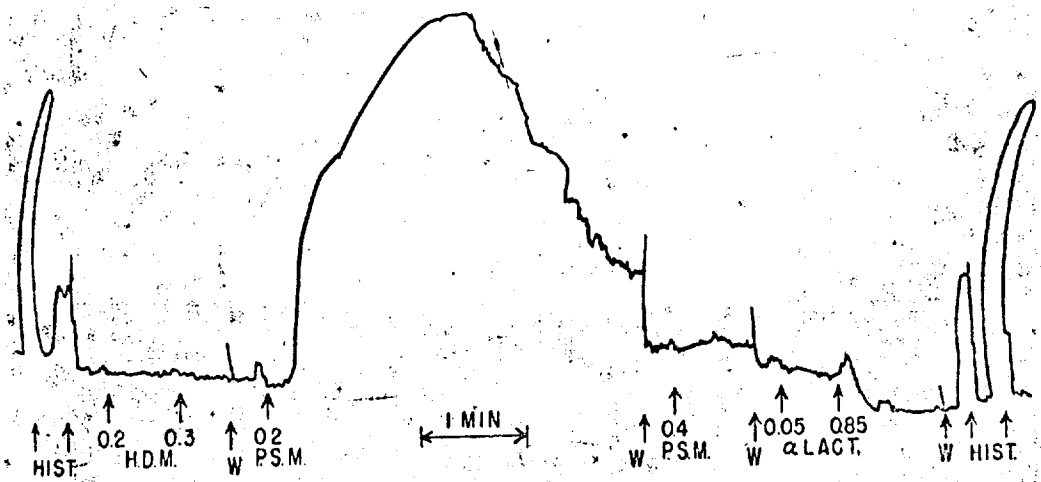

FIG. 2. In-vitro demonstration of heat lability of alpha-lactalbumin in heat-denatured milk. Segment of ileum from guinea pig sensitized to alpha-lactalbumin. From left to right: HIST. = 0.2 and 0.1 μg histamine, each followed by three washes; H.D.M. = 0.2 and 0.3 ml heat-denatured milk added to bath–no reaction (indicating that the lactalbumin has been denatured); W = three washes; P.S.M. = 0.2 ml pasteurized skimmed milk–typical anaphylactic reaction; 0.4 ml P.S.M.–no response (desensitization); α-LACT. = 0.05 and 0.85 mg alpha-lactalbumin with no response (desensitization); HIST. = 0.1 and 0.2 μg of histamine with three washes after each addition, demonstrating viability of segment.

denatured milk. However, heat-denatured milk produced almost no anaphylactic reactions when injected into animals sensitized to beta-lactoglobulin.

The alpha-casein fraction retained its allergenicity both as a sensitizing and shocking agent although there was a slight reduction of allergenicity when heat-denatured milk was used as a shocking agent with animals sensitized to casein. This suggests that casein may not be completely heat stable, although it has been considered so in the past.

It must be pointed out that the protein content of the heat-denatured milk used in these experiments was approximately one half that in the pasteurized skimmed milk, being 1.7 compared to 3.5 gm/100 ml. Thus, it is possible that, at least in part, the reduction of the allergenicity of heat-denatured milk may be due to its lowered protein content. However, it seems unlikely that the difference in dosage could be responsible for the striking differences in reaction to intravenous injection of heat-denatured milk and pasteurized skimmed milk in animals sensitized to alpha-lactalbumin and beta-lactoglobulin (Table II).

CONCLUSIONS

As demonstrated by parenteral sensitization and challenge in anaphylaxis experiments with guinea pigs, a particular heat-denatured milk was shown to have lost the allergenicity of the alpha-lactalbumin fraction, partially lost that of the beta-lactoglobulin fraction, and retained the allergenicity of the alpha-casein fraction.

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SUMMARIO IN INTERLINGUA

Studios Relative Al Allergenicitate De Lacte Bovin II.

Le presente studio esseva interprendite pro investigar le alterationes effectuate in le allergenicitate de alpha-lactalbumina, beta-lactoglobulina, e alpha-caseina de lacte bovin quando le lacte es processate a calor.

Le pur fractiones proteinic—alpha-lactalbumina, beta-lactoglobulina, e alpha-caseina—esseva describite in un previe reporto. Le pasteurisate lacte discremate, que esseva usate in certes de iste experimentos, esseva obtenite ab ordinari fontes commercial. Le lactes calefacite que es hic studiate va esser designate como "lacte thermodisnaturate" e representa varie preparatos de disponibilitate commercial que es calefacite ante lor evaporation.

Tests de anaphylaxe, utilisante porcos de India intacte e isolate segmentos del ileum de porcos de India, esseva executate como in le previemente reportate studio del presente serie.

Esseva constatate que le allergenicitate de alpha-lactalbumina esseva eliminate in le lacte thermodisnaturate. Isto esseva demonstrate tanto per utilisar le lacte como agente de sensibilisation como etiam per utilisar lo como stimulante in animales previemente sensibilisate con fractiones proteinic de lacte.

Le fraction beta-lactoglobulina, previemente recognoscite como un potentissime antigeno, retineva su qualitates sensibilisatori in le lacte thermodisnaturate. Tamen, lacte thermodisnaturate produceva quasi nulle reactiones anaphylactic quando illo esseva injicite in animales sensibilisate a lacto-globulina.

Le fraction alpha-caseina retineva su allergenicitate tanto como agente sensibilisatori como etiam como agente de stimulation. Le leve reduction de iste allergenicitate in lacte thermodisnaturate signala que caseina non es completemente thermo-stabile, ben que illo ha essite considerate como tal in le passato.

Le conclusion del presente studio es que le thermodisnaturate lacte de disponibilitate commercial—secundo experimentos anaphylactic a sensibilisation e stimulation parenteral de porcos de India—ha perdite le allergenicitate attribuibile al fraction alpha-lactalbumina, ha partialmente perdite le allergenicitate attribuibile al fraction beta-lactoglobulina, sed ha retenite le allergenicitate attribuibile al fraction alpha-caseina.

STUDIES ON THE ALLERGENICITY OF COW'S MILK

III. Effect of Heat Treatment on the Allergenicity of Milk and Protein Fractions from Milk as Tested in Guinea Pigs by Sensitization and Challenge by the Oral Route

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IN A PREVIOUS study[1] it was shown that a commercially available heat-denatured milk had lost the allergenicity of the alpha-lactalbumin fraction, partially lost that of the beta-lactoglobulin fraction, while it had retained the allergenicity of the heat stable alpha-casein. These findings were demonstrated by parenteral sensitization and challenge of guinea pigs.

In the present investigation we have attempted to relate these findings to the clinical problem of milk allergy by using the oral route for sensitization and challenge.

MATERIALS AND METHODS

The milk products used were previously described.[1, 2]

Oral sensitization and oral challenge were carried out according to the following procedure: after a 10-hour period of fasting, guinea pigs weighing 250 gm were immobilized in a cloth bag with the head exposed. The mouth was held open with a small clamp and the desired quantity of milk was introduced slowly through a pipette so that the animal swallowed without vomiting or choking.

RESULTS

Heat-denatured Milk and Pasteurized Skimmed Milk as Challenging Agents by the Oral Route

Three groups of 10 animals each received primary parenteral injections of alpha-lactalbumin, beta-lactoglobulin and alpha-casein (Table I, A, B, C). Three weeks later the animals were challenged orally with 5.0 ml of heat-denatured milk and, 2 hours later, with 5.0 ml of pasteurized skimmed milk.

None of the animals showed any reaction when challenged orally with heat-denatured milk. When they were subsequently challenged orally with pasteurized skimmed milk, one of the animals sensitized to alpha-lactalbumin showed a moderate reaction, one of the animals sensitized to beta-lactoglobulin exhibited mild symptoms and two of the animals sensitized to alpha-casein reacted, one mildly and the other with severe, nonfatal anaphylaxis. Thus, no reaction ensued in animals sensitized to the individual protein fractions of milk when they were challenged orally with heat-denatured milk, but when challenged orally with pasteurized skimmed milk, anaphylactic reactions occurred in four animals.

When these same animals were challenged 1 hour later with pasteurized skimmed milk intravenously, 25 manifested anaphylactic reactions, of which 15 were fatal.

Therefore, of these 25 animals proved hypersensitive to whole milk by parenteral challenge, four (16%) showed anaphylactic reactions after challenge with pasteurized skimmed milk by the oral route, while none of the 25 animals so reacted to oral chal-

[1] Posthumously submitted.

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TABLE I*

COMPARISON OF ALLERGENICITY OF HEAT-DENATURED MILK WITH PASTEURIZED SKIMMED MILK AS TESTED BY ORAL CHALLENGE

Animal	Parenteral Sensitization, 1 mg Sc + 1 mg Ip; 2 Days Later 1 mg Sc	Oral Challenge, 5 ml 21 Days Later: Material	Result	Material	Result	Parenteral Challenge, 0.1 ml Iv 1 hr Later: Material	Result
A 1	α-Lactalbumin	HDM	0	PSM 2 hr later	0	PSM	0
A 2			0		0		++++
A 3			0		0		+
A 4			0		0		+++
A 5			0		0		++++
A 6			0		0		++++
A 7			0		0		++
A 8			0		0		++++
A 9			0		0		0
A 10			0		++		++++
B 1	β-Lactoglobulin		0		0		0
B 2			0		0		++++
B 3			0		0		++++
B 4			0		0		0
B 5			0		0		++++
B 6			0		0		++++
B 7			0		0		++++
B 8			0		+		++++
B 9			0		0		++++
B 10			0		0		++++
C 1	α-Casein		0		+++		+++
C 2			0		+		++++
C 3			0		0		+
C 4			0		0		+
C 5			0		0		+
C 6			0		0		+
C 7			0		0		++++
C 8			0		0		++++
C 9			0		0		0
C 10			0		0		++

Animal	Parenteral Sensitization, 1 mg Sc; 28 Days Later 2 mg Sc	Oral Challenge, 5 ml 52 Days Later: Material	Result	Material	Result	Parenteral Challenge, 0.1 mg Iv 1 hr Later: Material	Result
D 1	α-Casein	HDM	0	PSM 2 hr later	0	α-casein	+
D 2			++++				
D 3			+		+++		++++
D 4			++		++++		
D 5			0		+		++++
D 6			0		++		++++

Animal	Parenteral Sensitization, 1 mg Sc; 42 Days Later 2 mg Sc	Oral Challenge, 5 ml 42 Days Later: Material	Result	Material	Result	Parenteral Challenge, 0.1 ml Iv: Material	Result
E 1	β-Lactoglobulin	HDM		PSM 2 hr later	+	PSM 1 hr later	+++
E 2			0		+		++++
E 3			0		+		++++
E 4			0		0		0
E 5			0		0		0
E 6			0		+		++++

* In this and subsequent tables:
Sc = subcutaneous; Iv = intravenous; Ip = intraperitoneal.
HDM = heat-denatured milk; PSM = pasteurized skimmed milk.
+ + + + = Anaphylactic death—dyspnea, convulsions, collapse, apnea, death.
+ + + = Severe anaphylaxis—dyspnea, convulsions, collapse, recovery.
+ + = Moderate anaphylaxis—dyspnea, convulsive movements, moderate collapse, recovery.
+ = Mild anaphylaxis—dyspnea and scratching.
0 = No reaction.

lenge with heat-denatured milk.

An additional six animals were then injected by a schedule different than above (Table I, D). They received 1.0 mg of alpha-casein subcutaneously and, 28 days later, a second subcutaneous injection of 2.0 mg. without reaction. Fifty-two days after the second injection, when they were given 5.0 ml of heat-denatured milk orally, three of the six animals had typical anaphylactic reactions, one of which was fatal. Two hours later, when the five surviving animals were challenged orally with 5.0 ml pasteurized skimmed milk, four had anaphylactic reactions, one of which was fatal. In every instance in which pasteurized skimmed milk caused a reaction, it was more severe than that manifested with heat-denatured milk, suggesting that, even though one animal died after ingestion of heat-denatured milk, there was a partial denaturation, even of the alpha-casein fraction. The four animals surviving oral challenge all showed anaphylactic response to parenteral challenge with 0.1 mg of alpha-casein administered intravenously. Three of these reactions were fatal.

Each of six animals received two subcutaneous injections of beta-lactoglobulin 27 days apart (Table I, E). Forty-two days after the second injection, oral challenge with 5.0 ml of heat-denatured milk caused no reaction. However, when these animals were challenged orally 2 hours later with pasteurized skimmed milk, mild anaphylactic reactions were noted in four of the six animals. After a parenteral injection of pasteurized skimmed milk 1 hour later, four animals died in anaphylaxis and two remained asymptomatic.

These data (Table I) show that of 42 animals, 35 were proved hypersensitive by oral challenge, parenteral challenge, or both.

Of these 35 hypersensitive animals, sensitized parenterally with one of the protein fractions of milk and challenged orally with heat-denatured milk, 3 (8.7%) showed anaphylactic reactions, one of which was fatal. However, all three reactions occurred in animals originally sensitized to alpha-casein, which is heat stable.[1] In contrast, 12 of the surviving 34 animals, or approximately 35%, showed anaphylaxis when challenged orally with pasteurized skimmed milk. One reaction was fatal (Table II).

TABLE II

INCIDENCE OF ANAPHYLACTIC REACTIONS TO HEAT-DENATURED MILK (HDM) AND PASTEURIZED SKIMMED MILK (PSM) FOLLOWING ORAL CHALLENGE IN HYPERSENSITIVE GUINEA PIGS

Sensitized with	*Challenged with*	
	HDM	*PSM*
α-Casein (15 sensitive)	3 15* (20%)	6 14 (42.8%)
β-Lactoglobulin (12 sensitive)	0 12 (0%)	5 12 (41.6%)
α-Lactalbumin (8 sensitive)	0 8 (0%)	1 8 (12.5%)

* Positive reactions/number of animals.

Heat-denatured Milk and Pasteurized Skimmed Milk as Sensitizing Agents by the Oral Route

Six animals were each fed 100 ml of heat-denatured milk over a three day period (Table III, A). Twenty-six days later they were challenged orally with 5.0 ml of pasteurized skimmed milk, with no reactions. One hour later each received 0.1 ml of pasteurized skimmed milk intravenously with no reactions.

Eight animals were each fed 100 ml of pasteurized skimmed milk over a three day period (Table III, B). Twenty-six days later, each received 5.0 ml of pasteurized skimmed milk orally. Five of the eight showed anaphylactic reactions, one of which was fatal. One hour later the surviving seven animals each received 0.1 ml of pasteurized skimmed milk intravenously and five showed anaphylactic reactions, three of which were fatal. Thus, six of eight animals had been sensitized by feeding pasteurized skimmed milk, five of which reacted on oral challenge.

Of nine animals fed 100 ml of pasteurized skimmed milk, none reacted when challenged orally 26 days later with 5.0 ml of heat-denatured milk (Table III, C). Two hours later each was challenged orally with

TABLE III

THE ALLERGENICITY OF HEAT-DENATURED MILK (HDM) AND PASTEURIZED SKIMMED MILK (PSM) AS TESTED BY ORAL SENSITIZATION AND ORAL AND PARENTERAL CHALLENGE

	Animal	Oral Sensitization, 100 ml over 3 Days	Oral Challenge, 5 ml 26 Days Later		Parenteral Challenge, 0.1 ml 1 hr Later
			HDM	PSM	PSM
A	1	HDM		0	0
	2			0	0
	3			0	0
	4			0	0
	5			0	0
	6			0	0
B	1	PSM		+	++
	2			+	++++
	3			0	0
	4			0	+
	5			+	++++
	6			++	++++
	7			0	0
	8			++++	
			(2 hr later)		
C	1	PSM	0	+	++
	2		0	+	++++
	3		0	+	++++
	4		0	0	++++
	5		0	0	+
	6		0	++	++
	7		0	0	++++
	8		0	0	+ + +
			0	+ +	+ + +

5.0 ml of pasteurized skimmed milk and five showed mild to moderate anaphylaxis. One hour later, intravenous injection of 0.1 ml of pasteurized skimmed milk resulted in anaphylaxis in all, four of which were fatal.

Thus, heat-denatured milk, administered orally, was found to be incapable of sensitizing animals, whether tested by oral or parenteral challenge. This is in striking contrast to the results obtained with animals fed pasteurized skimmed milk, for 15 of 17 (88%) were sensitized, as tested by oral or intravenous challenge, or both (Table IV). In these animals orally sensitized with pasteurized skimmed milk, oral challenge with heat denatured milk produced no reactions,

TABLE IV

SUMMARY OF TABLE III A, B, C

Sensitized with	Challenged with		
	HDM Oral	PSM Oral	PSM Iv
HDM Oral	0/6*	0/6	0/6
PSM Oral	0/9	10†/17	14/16

* Positive reactions/number of animals.
† One died in anaphylaxis.

whereas oral challenge with pasteurized skimmed milk caused reactions in 10 of 17 animals, or 60%, one of which was fatal.

COMMENT

Using immunologically pure fractions,[2] it has been demonstrated that heat-denatured milk has lost the allergenicity of the alpha-lactalbumin fraction, partially lost that of the beta-lactoglobulin fraction, and retained the allergenicity of the alpha-casein fraction.[4] These results were obtained through parenteral sensitization and challenge of guinea pigs and thus are not necessarily applicable to milk allergy in the human.

Therefore the present study was carried out, in which guinea pigs were sensitized and challenged by the oral route with heat-denatured milk and undenatured milk and milk fractions.

It should be noted that 100 ml of milk fed during a 3-day period to a guinea pig weighing 250 gm, as was done in these experiments, is about the same quantity per kilogram as that given to an infant weighing 3,600 gm.

The liquid form of heat-denatured milk was used in most of these studies, although preliminary experiments and some of the earlier studies[4] used the reconstituted dried form. In the past, milk was flash-dried directly from the raw form and retained much of its allergenicity.[5] The powdered milk used in these experiments was heated and condensed prior to the drying process[3] and, therefore, was essentially the same as liquid heat-denatured milk.

Under the conditions of this experiment, it was first shown that anaphylaxis could be produced by oral challenge with undenatured milk in hypersensitive animals, although in a much smaller percentage than by parenteral challenge.

In contrast, such reactions could be produced with heat-denatured milk given orally only in animals hypersensitive to the heat-stable alpha-casein.

The effect of heat denaturation was even more striking when attempts were made to sensitize guinea pigs by the oral route. While feeding of pasteurized skimmed milk resulted in sensitization of about two-thirds of the animals, as tested later by oral challenge, and in almost all of them subsequently tested by parenteral challenge, attempts to sensitize with heat-denatured milk administered orally resulted in no demonstrable sensitization, even when the animals were tested by parenteral (intravenous) challenge with pasteurized skimmed milk.

It should be pointed out that the heat-denatured milk used in these experiments had a protein content approximately one-half that of the pasteurized skimmed milk, being 1.7 gm/100 ml as compared with 3.5 gm/100 ml.[1] Therefore, a possibility exists that at least part of the decreased allergenicity of the heat-denatured milk may be due to this lowered content of protein. However, it seems unlikely that the striking differences between heat-denatured milk and pasteurized skimmed milk could be due to this difference in dosage.

CONCLUSIONS

Oral challenge with heat-denatured milk produced no reactions in guinea pigs sensitized parenterally to beta-lactoglobulin and alpha-lactalbumin, but produced typical anaphylactic reactions in animals sensitized to the heat-stable alpha-casein. In contrast, feeding of pasteurized skimmed milk resulted in frequent reactions in animals sensitized to alpha-casein and beta-lactoglobulin and one reaction in an animal sensitized to alpha-lactalbumin.

Oral administration of heat-denatured milk failed to produce sensitization, even as tested by intravenous challenge with pasteurized skimmed milk. In contrast, pasteurized skimmed milk, given orally, resulted in sensitization of about two-thirds of the animals, as tested later by oral challenge with pasteurized skimmed milk, and of an even greater proportion as tested by intravenous challenge.

Although alpha-casein is generally considered to be heat stable, under the conditions of these experiments the evidence indicated that it may be partially denatured by heat.

These studies, designed to stimulate clinical sensitization to milk, suggest that heat-denatured milk can be fed safely to individuals allergic to milk, unless they are extraordinarily sensitive to the heat-stable alpha-casein.

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SUMMARIO IN INTERLINGUA

Studios Relative Al Allergenicitate De Lacte Bovin III.

In un previe studio del presente serie, il esseva monstrate que un thermodisnaturate lacte de disponibilitate commercial habeva perdite le allergenicitate del fraction alpha-lactalbumina, partialmente perdite illo del fraction beta-lactoglobulina, e retenite illo del thermostabile alpha-caseina. Iste conclusiones esseva basate super le sensibilisation e stimulation parenteral de porcos de India.

In le presente investigation, le tentativa esseva interprendite de relationar le supra-

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Antigenicity of milk proteins of prepared formulas measured by precipitin ring test and passive cutaneous anaphylaxis in the guinea pig

The antigenicity of the major whey proteins and of casein in a variety of infant milk preparations has been measured by the precipitin ring and by the passive cutaneous anaphylaxis tests. All milk formulas showed casein, alpha-lactalbumin, and beta-lactoglobulin activity. Some products also showed bovine serum albumin activity. It is suggested that the term "heat denatured milks" be abandoned as a designation implying nonantigenicity or nonallergenicity of the proteins in processed milk preparations. The data suggest that the use of heat modified milks in the diet of patients allergic to casein, alpha-lactalbumin, or beta-lactoglobulin is contraindicated.

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THE term "heat denatured milk" appears quite frequently in the medical literature, especially in publications dealing with allergy, without a clear and accurate definition. Often, loss in allergenicity or antigenicity is implied when a writer discusses application of heat to modify various foodstuffs for an allergic individual. Certain milk products are generally included in this broad classification of "heat denatured" foodstuffs, e.g., evaporated milk, canned liquid-prepared infant formulas, and powdered-prepared infant formulas.

It seems important that the term "heat denatured" be fully understood and not be used synonomously with loss in allergenicity or loss in antigenicity.

As applied to proteins, the term "denaturation" includes any change in physical and chemical properties from the native state. Among the manifestations of denaturation are loss of solubility, changes in electrophoretic mobility, in number of SH groups, in viscosity, etc. Such changes can be brought about by heat, enzyme action, chemical and various physical treatments. Putnam[1] has

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presented an excellent review on protein denaturation.

When applied to milk, the term "heat denatured" does not necessarily indicate accurately the relative antigenicity of the protein moieties involved, since some of the denatured proteins retain to a large extent their antigenic properties even after extensive heating, e.g., alpha-casein, beta-casein, beta-lactoglobulin A, beta-lactoglobulin B.

The process of denaturation is not necessarily an all-or-nothing condition. With some proteins, such as the beta-lactoglobulins of cow's milk, some stages of heat denaturation are reversible.[2] A denatured protein may fail to show a precipitin reaction and still be capable of blocking a precipitin reaction between precipitating antibody and the native protein.[3] It is also known that some denatured proteins may produce antibodies to the denatured protein as well as to the unaltered protein.[4]

The purpose of this paper is to clarify the relationship of canned and dried milks to the relative antigenicity and/or allergenicity of their proteins.

The determination of allergenicity of prepared infant formulas for infants and children having milk allergy will be presented in a later publication.

The three whey proteins investigated in this study were chosen because together they represent the major portion of the total whey proteins. These proteins have also been demonstrated to produce clinical symptoms of milk allergy in milk-sensitive individuals. They also lend themselves more readily to isolation and purification than the remaining proteins of milk.

While there are other minor proteins indigenous to milk which warrant investigation as allergens, such studies have to be put in abeyance until adequate methods for their isolation and purification are available.

EXPERIMENTAL

Proteins. Alpha-lactalbumin, prepared by the methods of Aschaffenburg and Drewry[5] and Gordon and Ziegler,[6] was crystallized 6 times before use in the production of specific rabbit antiserum. It was free of beta-lactoglobulin and bovine serum albumin (BSA) as tested by agar gel electrophoresis and the precipitin reaction; however, by immunoelectrophoresis,[7] it showed a trace of BSA.

Beta-lactoglobulin, prepared by the method of Larson and Jenness,[8] was crystallized 6 times. It was free of alpha-lactalbumin as determined by the preceding techniques. This protein, prepared from a pooled milk source, contained both A and B forms of beta-lactoglobulin.[9]

Casein was prepared by precipitation from pasteurized skim milk at 70° F. (21° C.) and pH 4.5, with acetic acid. It was subsequently reprecipitated 10 times at pH 4.5. The casein was washed after each precipitation. Ammonia was used to dissolve the pH 4.5 precipitated casein. The solubilized casein was brought to an approximate concentration of 0.8 per cent weight per volume prior to reprecipitation.

Bovine serum albumin* was found to contain a small amount of bovine gamma globulin (BGG). This contaminant, however, did not interfere with the test procedures.

Immunologic methods. The method for production of the rabbit antisera to the various proteins has been described.[10] Antibody nitrogen (AbN) levels were measured by the quantitative precipitin method.[11] The antigenicity of several commercially prepared infant milks was determined by the precipitin ring test[10, 12] and by passive cutaneous anaphylaxis (PCA) in the guinea pig.[13]

The PCA test. Guinea pigs, male and female, weighing approximately 300 grams, were injected intradermally with 0.1 ml. of specific antisera with the use of a 1 cm. (3/8 inch) long 26 gauge needle, along both sides of the back. A total of 6 sites, 2 for each antiserum, were used. Three animals were used for each milk tested. The rabbit antisera were previously diluted with 0.87 per cent weight per volume of sodium chloride. Six hours later, 0.5 ml. of 1 per cent weight

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Table I. Antigenicity of alpha-lactalbumin and beta-lactoglobulin as determined by precipitin tests of several reconstituted liquid infant milk formulas after terminal heating at 110° C. (230° F.) for 10 minutes

	*Antigen dilution**					
Liquid products	*1/500*	*1/1,000*	*1/2,000*	*1/4,000*	*1/8,000*	*Serum*
Enfamil	+	+	†	–	–	A (anti-alpha-lactalbumin)
	+	+	+		–	B (anti-beta-lactoglobulin)
Modilac	+	+	+	–	–	A
	+	+	+	+	–	B
Lactum	+	+	–		–	A
	+	+	+	–	–	B
Similac	+	+	+	–	–	A
	+	+	+	+	–	B
Bremil	–	–	–	–	–	A
	+	+	+	–	–	B
SMA	+	+	+	+	–	A
	+	+	+	+	+	B
Bakers Modified	+	+	–	–	–	A
	+	+	+	+	–	B

*Antigen dilution is based on approximate protein content remaining in the whey after precipitation of the casein at pH 4.5 (N × 6.38 = 0.4% for starting material).

†Reaction time was carried on for 180 minutes before being called negative.

per volume of Evan's blue in 0.87 per cent weight per volume of sodium chloride was injected intravenously into the animal. Appearance of a blue spot at the injection site within 20 minutes indicated a nonspecific response and such animals were eliminated from the test. Nonspecific reactions, however, seldom occurred. The protein challenge dose was then given each animal. The dose consisted of 1.0 ml. of milk product previously diluted with an equal volume of 0.87 per cent weight per volume of sodium chloride. The challenge dose was also given intravenously.

After 15 minutes, the animals were sacrificed and the underside of the skin was examined. Increased capillary permeability is a consequence of an anaphylactic reaction in the skin. A positive reaction results in leakage of the blue dye from the capillaries into the surrounding area.

For use in the precipitin test, all milk products were reconstituted to 20 calories per 30 ml. (1 fluid ounce) and terminally heated. One group of reconstituted formulas was heated under pressure for 10 minutes at 110° C. (230° F.) and a second group heated for 30 minutes at 99° C. (210° F.). Following this, all formulas were adjusted to contain 1.5 Gm. of protein per 100 ml. The whey proteins were tested as described previously,[10] with the use of the ring precipitin test after removal of casein at pH 4.5. The liquid and powdered milk products examined were purchased from retail outlets.

RESULTS

Tables I and II show the antigenicity by precipitin ring test of the liquid infant milk preparations against antisera prepared for beta-lactoglobulin and alpha-lactalbumin. All samples tested were positive to beta-lactoglobulin and alpha-lactalbumin antisera.

The data in Tables I and II show that some of the infant formula preparations contain more residual reactive whey proteins than others, and that not one of the preparations is devoid of reactive alpha-lactalbumin or beta-lactoglobulin. The differences found among the various milks may be a reflection of the total heat treatment given each milk product throughout the manufacturing process.

Table III shows the results obtained with powdered infant formulas by the use of the ring test. Powdered products receive less total heat during manufacture than liquid products. As was expected, these lower heat

Table II. Antigenicity of alpha-lactalbumin and beta-lactoglobulin as determined by precipitin tests of several reconstituted liquid infant milk formulas after terminal heating at 99° C. (210° F.) for 30 minutes

	*Antigen dilution**					
Liquid products	*1/500*	*1/1,000*	*1/2,000*	*1/4,000*	*1/8,000*	*Serum*
Enfamil	+	+	−†	−	−	A (anti-alpha-lactalbumin)
	+	+	+	−	−	B (anti-beta-lactoglobulin)
Modilac	+	+	−	−	−	A
	+	+	+	+	−	B
Lactum	+	+	+	−	−	A
	+	+	+	+	−	B
Similac	+	+	+	−	−	A
	+	+	+	+	+	B
Bremil	+	+	−	−	−	A
	+	+	+	+	−	B
SMA	+	+	+	−	−	A
	+	+	+	+	+	B
Bakers Modified	+	+	+	−	−	A
	+	+	+	+	+	B

*Antigen dilution is based on approximate protein content remaining in the whey after precipitation of the casein at pH 4.5 (N × 6.38 = 0.4% for starting material).

†Reaction time was carried on for 180 minutes before being called negative.

products showed more residual activity of alpha-lactalbumin and beta-lactoglobulin than the canned liquid milks.

The results of the PCA reactions are shown in Table IV. All of the products were positive for alpha-lactalbumin, beta-lactoglobulin, and casein whether or not the formulas were subjected to terminal heating.

Most of the powdered infant foods gave a positive test for BSA before terminal heating, but only 2 (Similac and Enfamil) retained their BSA activity following terminal heating for 30 minutes at 99° C. (210° F.).

The effect of heat on these whey proteins in fresh skim milk is given in Table V. Although BSA is inactivated in 45 minutes at 99° C. (210° F.), 180-minute heating at 99° C. (210° F.) did not inactivate the alpha-lactalbumin and beta-lactoglobulin as tested by the PCA reaction.

Table III. Antigenicity of alpha-lactalbumin and beta-lactoglobulin as determined by precipitin tests of several reconstituted powdered infant milk formulas after terminal heating at 99° C. (210° F.) for 30 minutes

	*Antigen dilution**						
Powder products	*1/500*	*1/1,000*	*1/2,000*	*1/4,000*	*1/8,000*	*1/16,000*	*Serum*
Enfamil	+	+	+	+	+	+	A (anti-alpha-lactalbumin)
	+	+	+	+	+	+	B (anti-beta-lactoglobulin)
Lactum	+	+	+	+	+	+	A
	+	+	+	+	†		B
Similac	+	+	+	+	+	+	A
	+	+	+	+	+	+	B
Bremil	+	+	+		−		A
	+	+	+				B
SMA	+	+	+	+	+	+	A
	+	+	+	+	+	+	B
Bakers Modified	+	+	+	+	+	+	A
	+	+	+	+	+	+	B

*Antigen dilution is based on approximate protein content remaining in the whey after precipitation of the casein at pH 4.5 (N × 6.38 = 0.4% for starting material).

†Reaction time was carried on for 180 minutes before being called negative.

Table IV. Antigenicity of bovine serum albumin, alpha-lactalbumin, beta-lactoglobulin, and casein of reconstituted milk formulas as determined by passive cutaneous anaphylaxis in guinea pigs*

Products†	*Anti BSA (0.13 μg AbN/ml.)*	*Anti alpha-lactalbumin (0.046 μg AbN/ml.)*	*Anti-beta-lactoglobulin (0.023 μg AbN/ml.)*	*Anti-casein (0.008 μg AbN/ml.)*
Liquid				
Bremil		+	+	+
SMA		+	+	+
Enfamil		+	+	+
Modilac		+	+	+
Lactum		+	+	+
Similac		+	+	+
Bakers		+	+	+
Powdered				
Bremil	–	+	+	+
SMA	+	+	+	+
Enfamil	+	+	+	+
Lactum	+	+	+	+
Similac	+	+	+	+
Bakers	+	+	+	+

*Skin sites sensitized with 0.1 ml. of diluted specific antiserum (rabbit). Antibody N content is an approximate value.

†All reactions were identical after terminal heating of reconstituted formulas at 99°C. (210°F.) for 30 minutes, except SMA powdered, Lactum powdered, and Bakers powdered, which became BSA negative.

Table V. Antigenicity of bovine serum albumin, alpha-lactalbumin, and beta-lactoglobulin of fresh skim milk as determined by passive cutaneous anaphylaxis after heating the milk at 99° C. (210° F.) for 3 hours.*

Heating time (minutes)	*Anti-BSA (0.13 μg AbN/ml.)*	*Anti-alpha-lactalbumin (0.046 μg AbN/ml.)*	*Anti-beta-lactoglobulin (0.023 μg AbN/ml.)*
30	+	+	+
45	–	+	+
180	–	+	+

*Skin sites sensitized with 0.1 ml. of diluted antisera (rabbit).

The antigenicity of casein, alpha-lactalbumin, beta-lactoglobulin, BSA, and BGG, as determined by PCA, of all the milk products examined is shown in Table VI. Regardless of the method of manufacture and the heat treatment given, all the milk products revealed casein, alpha-lactalbumin, and beta-lactoglobulin to be active. Dried instant skim milk and pasteurized milk showed BSA and BGG to be active. All but one of the powdered infant formulas showed BSA activity.

Cross reactions were obtained only between BSA and alpha-lactalbumin when the respective antisera were used at dilutions less than 1:250 (amounts greater than 0.13 μg AbN ml.).

DISCUSSION

These data indicate that the antigenic determinants of casein, alpha-lactalbumin, and beta-lactoglobulin are not inactivated by the heat given to commercial milk products during manufacture. BSA, found to be inactive in canned liquid milk products, was active in powdered infant milk formula products. BGG activity was detected only in milks which had not been subjected to high heat, e.g., spray dried skim milk and fresh pasteurized milk. Thus it can be seen that heat denaturation of milk is not an all inclusive process.

The previous assumption[14-16] that the antigenicity or allergenicity of alpha-lactalbu-

Table VI. Protein antigenicity of milk products measured in vivo by passive cutaneous anaphylaxis in the guinea pig.

Milk product	Antigenicity of proteins				
	Casein	Alpha-lactalbumin	Beta-lactoglobulin	BSA	BGG
Evaporated milk	+	+	+	–	–
Dried instant skim milk (Starlac)	+	+	+	+	+
Pasteurized fluid cow's milk	+	+	+	+	+
Prepared infant formulas					
Liquid Similac	+	+	+	–	
Liquid Bakers	+	+	+	–	
Liquid Bremil	+	+	+		
Liquid SMA	+	+	+	–	
Liquid Enfamil	+	+	+	–	
Liquid Modilac	+	+	+	–	
Liquid Lactum	+	+	+	–	
Powdered Similac	+	+	+	+	–
Powdered Bremil	+	+	+	–	
Powdered SMA	+	+	+	+	–
Powdered Enfamil	+	+	+	+	
Powdered Lactum	+	+	+	+	–
Powdered Bakers	+	+	+	+	–

min, beta-lactoglobulin is destroyed by boiling milk, evaporating milk, or heat processing canned milk formulas, is not borne out with the use of the techniques described earlier.

The heating of milk does result in certain changes in many of the milk proteins, such as rupture of -S-S-linkages. This cleavage does not, however, necessarily alter the immunologic activity of the protein. Maurer and Heidelberger[1] found little change in immunologic specificity of egg albumin resulting from a -S-S- to SH shift. Harland, Coulter, and Jenness[17] and Jenness[18] showed an activation of the -SH bonds when milk is heated. The data presented here indicate that this shift of -SH to -S-S- in beta-lactoglobulin does not significantly reduce its antigenicity.

The report of Brown, Aurand, and Roberts,[19] as well as our own unpublished results on the electrophoretic patterns of heated milks, demonstrated a reduction in the amount of alpha-lactalbumin and beta-lactoglobulin when milk is heated under various conditions. However, we have found a significant amount of these proteins remain in heated milks. In work to be reported at a later date clinical evidence will be presented showing the allergic response of milk-sensitive children to heated milk formulas. It will also be demonstrated that only a few milligrams of the whey proteins are required to elicit an allergic response in some milk-sensitive patients.

The problems which have arisen in the establishment of the basic criteria for defining milk allergy, as well as its treatment, are in some measure the result of incomplete or inadequate experimental and clinical data. While many of the early studies in this field were handicapped by the lack of sufficiently pure milk proteins, some of the more recent studies must be closely questioned if not invalidated due to the omission of suitable controls in the design of the experiments.[11, 14, 21]

The data reported here are inconsistent with those of Crawford[11, 12] and Ratner and colleagues.[20] In Crawford's work,[11] adequacy of heating as an allergen denaturing agent was based on the use of a single human serum for a single protein. In each event proof of allergy to the purified protein was based on skin tests. Such tests are inadequate inasmuch as the patient from whom the serum was obtained was not orally challenged with the purified protein or with

the so-called heat denatured milk product.* Also, should this individual patient fail to respond to the heat denatured milk, it would indicate only that the degree of sensitivity was low enough to cope with the challenge. It certainly does not indicate that a large number of individuals allergic to milk would similarly fail to respond to such a challenge dose.

The sera obtained from these milk-allergic children were not tested for antibodies to BSA and BGG. These two proteins have been shown to give rise to many allergic reactions in children.[21]

In a clinical study currently in progress, we have found no correlation between the scratch or intradermal test and the test feeding of the same purified proteins. Ratner[22] found that the Prausnitz-Kustner test was equally unreliable.

It is, therefore, reasonable to assume that the differences which Crawford[14] found between pasteurized and heated milk could have resulted from inactivation of BSA or BGG, 2 of the more heat-sensitive proteins in milk, and not necessarily from the inactivation of alpha-lactalbumin and beta-lactoglobulin.

In a further continuance of this work, Crawford and Grogan[17] claim to show an inactivation of the heat stable protein, alpha casein. Our results as well as those of Hanson and Mansson[23] do not reveal such inactivation. Since no control was run in the work presented by Crawford and Grogan to demonstrate that their antiserum for alpha-casein could produce a precipitin band with the homologous protein, their results are highly questionable. The precipitin lines which they showed do not have the shape or position which a molecule as small as alpha-casein would be expected to produce due to its rapid diffusion characteristics. The precipitin lines which they demonstrated would appear to have been produced from a higher molecular weight protein contaminant. This certainly requires further study.

A careful analysis of the data presented by Ratner and his associates[20] shows that their animals, when sensitized by parenteral injection of protein, did not respond well to oral challenge of milk or milk proteins. They did not, however, challenge these guinea pigs by intravenous injection of their heat denatured milk product. In a prior work by the same authors,[24] their challenge of sensitized guinea pigs with heat denatured milk appeared to show loss in antigenic activity for beta-lactoglobulin and alpha-lactalbumin. The fact that they used an amount of protein equal to or less than their sensitizing dose when challenging with the heated milk product would tend to invalidate their negative results. Our own tests on the same product* were described earlier[10] and it was shown that sufficient alpha-lactalbumin and beta-lactoglobulin were present to cause anaphylactic death in adequately sensitized guinea pigs.

Furthermore, in the work of Ratner and his co-workers[24] they attempted to prove by means of the Schultz-Dale test that alpha-lactalbumin was destroyed in their heated milk. In this test, however, they presumed that their guinea pigs were sensitized to alpha-lactalbumin but showed no evidence of such sensitivity. Their kymograph tracing merely revealed that the guinea pig ileum did not respond to heated milk whereas it did respond to pasteurized skim milk. No control was run for alpha-lactalbumin. Since alpha-lactalbumin is most commonly contaminated with BSA and several samples of alpha-lactalbumin obtained from the same source† was shown to contain BSA,[25] it is very likely that these workers were getting a sensitization to BSA in their animals and not to alpha-lactalbumin. The BSA is more heat sensitive and, therefore, would show a negative test in their experimental procedure if liquid Similac was used.

The present data reconfirm our earlier findings as to the heat stability of the anti-

*Similac.

*Similac.

†Supplied by Dr. T. L. McMeekin and Dr. W. G. Gordon, Eastern Utilization Research and Development Division, United States Department of Agriculture, Philadelphia, Pa.

genic determinant groups of alpha-lactalbumin, beta-lactoglobulin, and casein.[10] Recently, the presence of all the aforementioned milk proteins were demonstrated in a number of foreign dried infant milk formulas by Hanson and Mansson[23] using the immunoelectrophoretic technique. While they could not demonstrate the presence of the whey proteins from milks heated to 120° C. (248° F.) for 15 minutes, this may reflect the sensitivity limits of the immunoelectrophoretic method. The PCA test is considered to be a more sensitive test.

Heating milk can render certain whey proteins (BSA and BGG) antigenically inactive and may reduce somewhat the antigenicity of alpha-lactalbumin. However, the heat processing given the milk products tested did not reduce the antigenicity of casein and/or beta-lactoglobulin. Therefore, it seems that the term "heat modified" would be a more accurate term to describe the antigenicity of heated intact milk proteins than "heat denatured." It has been shown that even among similar heat modified milk products that the total heat treatment given during manufacture differs.

On the basis of the information presented here, it would appear imprudent to prescribe any form of heated milk in known instances of casein allergy. The use of heat modified milks, wherein BSA and BGG have been inactivated, could well be used for children known to be allergic to either of these two proteins. Since the allergens alpha-lactalbumin and beta-lactoglobulin are not totally inactivated during the processing of milk or milk products, it is likely that in many instances of milk allergy, the feeding of such products would result in an allergic response.

SUMMARY

The antigenicity of the major whey proteins and of casein in a variety of milk products has been demonstrated by the precipitin ring and by the passive cutaneous anaphylaxis tests. All milk formulas showed casein, alpha-lactalbumin, and beta-lactoglobulin activity. Some products also showed bovine serum albumin activity. It is suggested that the term "heat denatured milks" be abandoned as a designation implying nonantigenicity of the proteins in processed milk preparations.

We wish to thank Dr. Nancy H. Holland for her aid in checking one of our alpha-lactalbumin preparations and Dr. Z. Ovary for his instructions in setting up the CPA test. We also wish to acknowledge the able technical assistance of Evelyn M. Cary.

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Erratum. In the article, "The physical development of the premature infant," by Frank Falkner, Alex J. Steigman, and Mary O. Cruise, in the June, 1962, issue of the JOURNAL, on page 897, the legends for Figs. 3 and 4 were reversed—Fig. 3 shows the gastrostomy device and Fig. 4 shows the typical appearance of fat deposition.

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Nutritional Blindness in the Cat

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Cats fed on a semi-purified diet containing casein developed classical signs of vitamin A deficiency in 6 – 20 months in spite of receiving oral supplements of vitamin A which have proved more than adequate on other types of diet. The diet resulted in conjunctivitis, xerosis with keratitis and vascularization of the cornea, photophobia, dilatation of the pupil in ordinary light, delay in the pupillary response to light, progressive destruction of the visual cells of the retina, and the formation of cataracts. These changes were associated with a marked reduction in the normally very high vitamin A content of the kidney and a fall in the liver reserve compared with cats on the stock diet. Addition of riboflavin and other B vitamins did not reverse the conjunctivitis and corneal vascularization. Reduction in the reserves of vitamin A was more rapid and complete when sucrose was the carbohydrate employed than when dextrin was given. Alteration in the type of fat, which formed 22% of the dry weight of the diet, did not affect the onset of the syndrome. While failure to absorb vitamin A could have brought about the changes observed it is more probable that it was the continual feeding of casein which made it difficult for the cat to utilize vitamin A. When vitamin A deficiency was induced on a meat diet no evidence was obtained of retinal damage, although conjunctivitis eventually appeared in kittens reared and maintained on the deficient diet.

1. Introduction

Investigations into the dietary requirements of the cat have been carried out by the authors over the past decade. In the course of these studies a semi-purified diet containing casein was devised, similar to the type of diet used in studying the requirements of the rat, but adapted to the known special requirements of the cat for a high protein, high fat diet. However, during the course of our investigations, defects of vision were observed in our animals. This paper described the investigations that arose from this observation.

2. Methods

A semi-purified diet, consisting of vitamin-free casein, saturated and unsaturated fats, and either sucrose or dextrin was mixed in the proportions shown in Table I. A standard salt mixture, modified from Hubell, Mendal and Wakeman (1937) was added at the 2% level, and vitamins were provided by adding a proprietary compound designed for dogs and cats (Nutricon, Bob Martin Ltd.); additional choline chloride and inositol were found to be essential, as in Table I (Greaves, 1959). Additional vitamin A was supplied as the palmitate (Glaxo) in an emulsion containing 10,000 IU/ml. The dry constituents of the diet were mixed about once a fortnight and stored in air-tight jars. The diet was fed ad libitum as a thick cream, mixed with distilled water. Intakes on this diet varied between 30 and 50 g dry diet/cat per day and compared favourably with those on other diets.

The cats were maintained in specially designed cages, usually two or more together, but individually when measuring food intake. General management and the composition of the stock and meat diets given to control cats have been described elsewhere (Dickinson and Scott, 1956; Scott, Greaves and Scott, 1961).

Eyes were examined regularly with an ophthalmoscope and, on occasion, with a slit lamp; behaviour and the time taken for the pupillary reaction to light were recorded. Vision was tested by allowing the cats to jump off a low stool.

Vitamin A estimations, by the antimony trichloride method after suitable extraction, on the diets, liver, kidneys and plasma were carried out in the early experiments by Dr. Moore

TABLE I

Ingredients	g/100 g dry diet
Casein (vitamin free)	35
Sucrose *or* dextrin	36·7
Lard	10
Arachis oil, Violmul K *or* cocoanut oil	12
Sugar beet residue	3
Salt mixture	2
Nutricon (vitamin supplement)	1
Choline chloride	0·3
Inositol	0·2

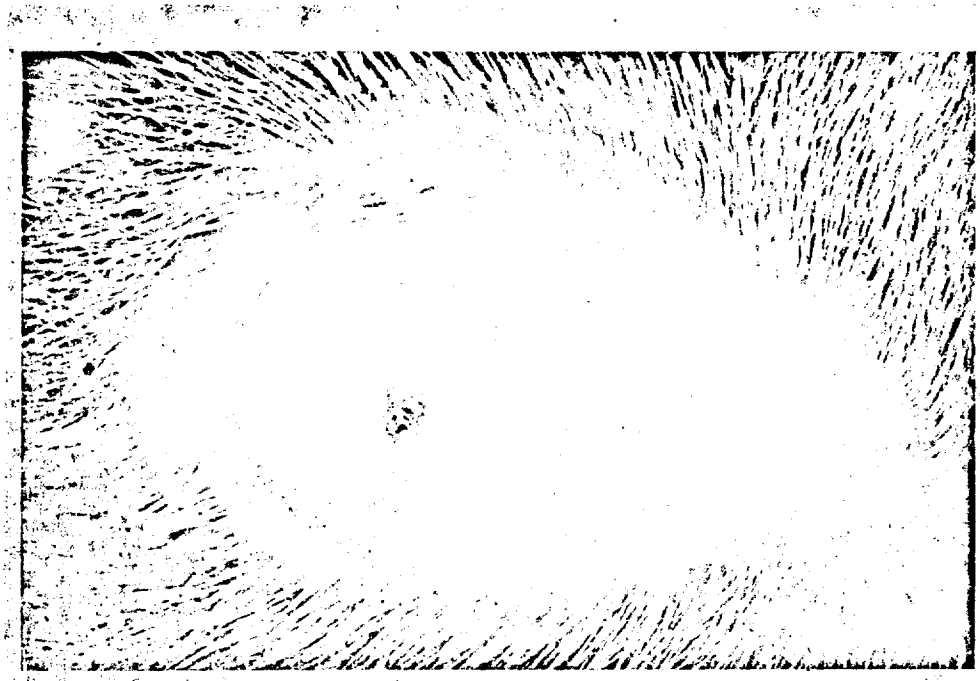
and Dr. Sharman of the Dunn Nutritional Laboratory, Cambridge, and in the later experiments by Mr. A. C. I. Shearer of the Research Department, Birmingham and Midland Eye Hospital. Eyes were fixed by Kohler's method or formal-saline. Fixation was begun by perfusion, under nembutal, and completed by immersion of the eyeball after removal by dissection. It was double-embedded in celloidin and paraffin wax, and sections were stained with Heidenhain's haematoxylin and Van Gieson, or haematoxylin and eosin. Sections in the last experiment were made by Dr. Barry, Regional Pathologist of the Birmingham and Midland Eye Hospital.

3. Results

Effect of feeding the casein–sucrose diet to an adult cat

An adult cat was reared and maintained on stock diet for 22 months, when it weighed 3·8 kg. Transferred to the casein–sucrose diet it did fairly well at first, but by 6 months had lost 29% of its weight. Its fur was unkempt and falling. In normal light its pupils were dilated, and the pupillary reflex to light very slow and incomplete. It began to bump into obstructions, and would not jump down from a stool 1 ft high. Keratitis and corneal vascularization developed (Plate 2(a)). The retinal vessels became attenuated until almost invisible.

Attempts were made to alleviate the cat's condition by giving vitamin A palmitate by mouth, and riboflavin and "B fortiss" by injection (Vitamins Ltd., containing aneurin 10 mg, riboflavine 1·mg, nicotinamide 40 mg and pyridoxine 1 mg), but no immediate response was obtained, as would be expected in rats deficient in one or more of these substances. After 8 months on the casein–sucrose diet the cat was in such poor condition that it was decided to change its diet completely. It was given raw meat supplemented with salts of calcium, and iodine and vitamin A palmitate (Scott et al., 1961). On this it rapidly regained weight and its fur, skin and cornea returned to normal, although it remained blind, with no obvious improvement in retinal blood supply. At post mortem the retina showed loss of visual elements, with disappearance of the outer nuclear layer (Plate 3(b)) suggesting the type of degeneration seen in vitamin A-deficient rats (Tansley, 1933; Johnson, 1939; Dowling and Gibbons, 1961). In spite of the severity of the retinal lesion, this cat still had a pupillary reflex to light, even though very delayed and incomplete.



(a)

(b)

PLATE I. (a) Cat A_2, Table III. Eye showing conjunctivitis, porphyrin exudate, xerosis and corneal vascularization at termination.

(b) Cat G_1, Table IV. Section of lens showing cortical cataract (× 100).

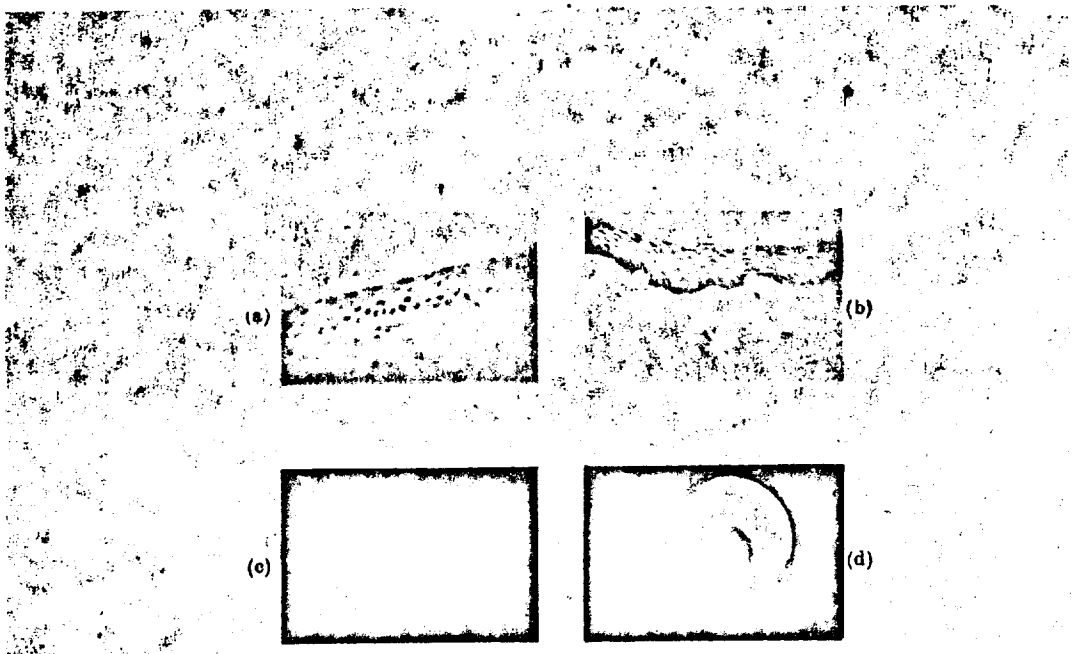


PLATE 2

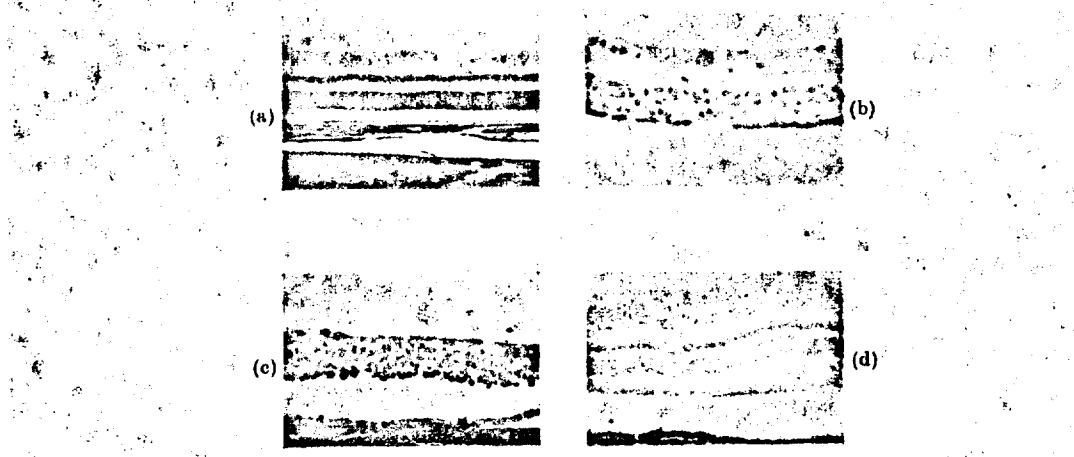


PLATE 3

PLATE 2. (a) Cornea from cat in first experiment after recovery on meat diet, epithelium normal but vascular channels still apparent in substantia propria (iron haematoxylin and Van Gieson) (× 220).

(b) Cornea from cat F, Table IV, showing squamous metaplasia of epithelium (haematoxylin and eosin) (× 220).

(c) Normal fundus of cat.

(d) Fundus of cat E. Table II showing swollen disc, diminution of blood vessels and loss of colour

PLATE 3. Sections of retina stained iron haematoxylin and Van Gieson.

(a) Normal (× 120). (b) Cat in first experiment showing loss of visual cells (× 220). (c) Cat B, Table III, showing degenerative changes (× 220). (d) Cat G_1, Table IV. Very early changes in visual elements (× 280)

To determine whether cats on the casein–sucrose diet were deficient in vitamin A

Fifteen kittens were weaned on to the casein–sucrose diet: 9 received 100 – 200 IU/day vitamin A in the diet according to the amount of food they ate; the remainder were further supplemented by giving 5,000 IU vitamin A palmitate orally three times weekly, equivalent to an average intake exceeding 2,000 IU/cat per day.

The kittens, especially those with the higher vitamin intake, grew well until they were from 3 to 4 months old, as compared with controls fed on a fresh meat diet supplemented with calcium and about 1,000 IU vitamin A/day. A decline in growth rate then occurred, more obvious in males than females (Fig. 1(a), (b)); both sexes on the casein–sucrose diet reached a plateau some 20–40% below their expected body weight. At post mortem, which was carried out at various ages between 4 and 21 months, these animals usually had reasonable stores of subcutaneous and abdominal fat. Skeletal muscles, in contrast, were reduced more than 50% below their expected weight for age, and some 30% below their expected weight expressed as a percentage of body weight at termination; that is, compared with cats of identical weight on other diets. Liver and kidney weights were also reduced, and, compared with cats of similar age on the stock diet (Table II), both the cats on the low intake and those receiving additional vitamin A had low values for vitamin A in the kidneys and liver. Although the

TABLE II

Vitamin A in liver and kidney on different diets (from Moore, Sharman and Scott, 1963)

Diet and vitamin A intake in IU/day	Number of kittens	Vitamin A IU/g Liver ± S.E.	Kidney ± S.E.
Casein-sucrose > 200	9	10± 5	33 ± 11
Casein-sucrose > 2000	5	470 ± 132	25 ± 8
Stock ≏ 1900	7	1900 ± 486	110 ± 29

additional vitamin significantly improved the reserves in the liver, they were not equal to those on the stock diet, especially when the discrepancy in the weight of the organ is taken into account. The oldest cats on this diet with both high and low intakes of vitamin A became blind and showed other signs that have been associated with vitamin A deficiency in rats, dogs and human beings. Briefly, these included conjunctivitis with the production of a dark reddish discharge, due to porphyrin production by the lacrimal glands (Cole and Scott, 1954; McLaren, 1963), keratitis, and corneal vascularization starting at the limbus which became progressively more apparent with time (Plate 1(a)); a foul mouth due to failure of mucous secretion, a characteristic inflammation of the gums and squamous metaplasia of the opening of the parotid duct.

At the onset of visual disturbance the cats showed a transient but severe photophobia which made examination of the retina difficult at this stage. Later, as the pupillary reflex became more and more delayed (up to 10 sec) and incomplete, examination became easy. The optic disc became swollen and the retina "fluffy" and occasionally slightly pigmented at the periphery. The retinal vessels became more and

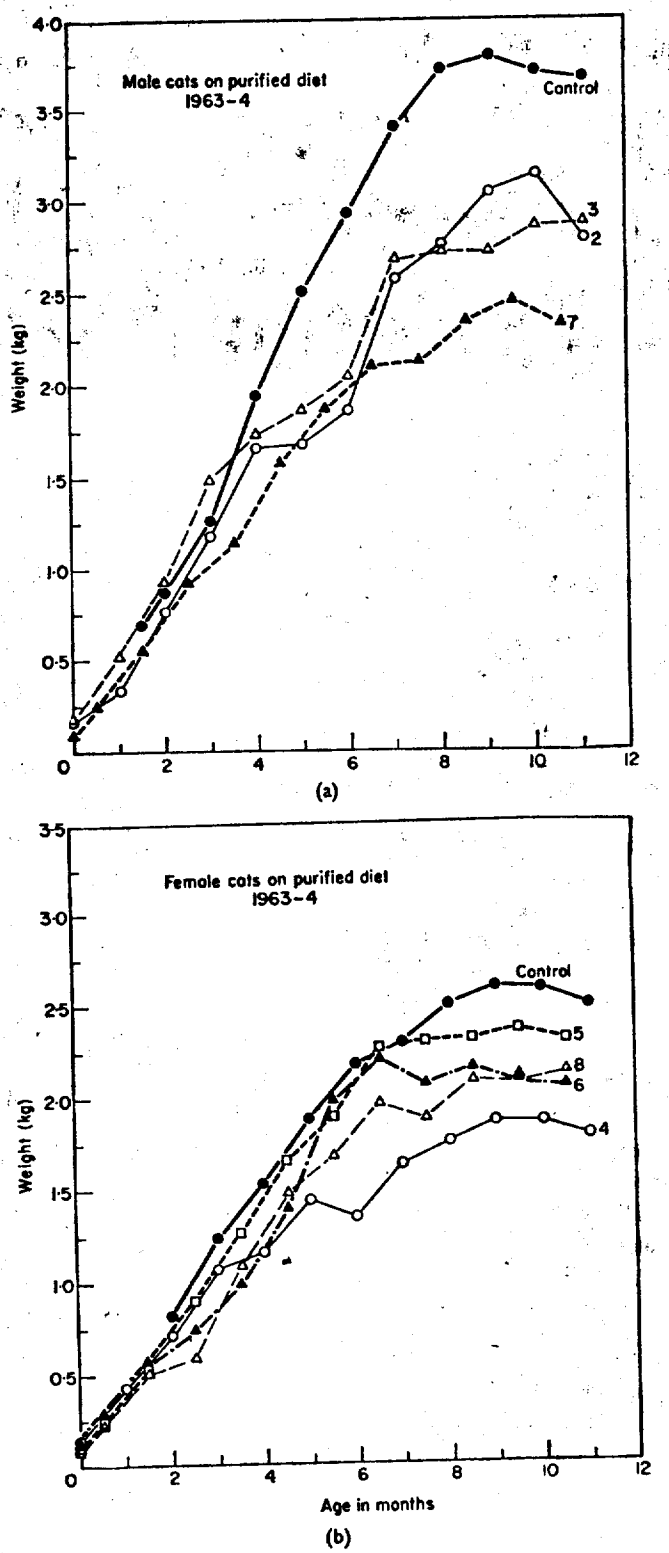


FIG. 1. (a) and (b) Growth curves of individual cats receiving 2000 IU Vitamin A palmitate per day Controls (solid circles and lines) fed on meat supplemented with $CaCO_3$, the others on semi-purifie casein based diet. (a) Males, (b) females.

more attenuated, and the retina paler in colour (Plate 2(c) and (d)). Sections of the retina of severely affected older cats showed loss of the visual cell elements, including their nuclei.

Effects of varying the constituents of the diet

Observations were made on the effect of varying the fat, carbohydrate and protein constituents of the semi-purified diet on the lesions aparently due to vitamin A deficiency observed in the previous experiment, and also on the ability of the liver and kidney to store the vitamin.

Eight kittens were reared on the casein–sucrose diet, with vitamin A palmitate mixed in the food to give 1000–1800 IU/day according to the amount of food eaten. In place of the lard and arachis oil previously given, four received highly saturated fats (palm oil provided by Unilever Ltd.) and four stabilized unsaturated fats (Violul K) fed at the 22% level as before. After 18 months and at termination no differences could be established between these groups.

After 18 months on the casein–sucrose diet the carbohydrate was changed to dextrin. The cats ate more and maintained their weights somewhat better on the casein–dextrin diet (Greaves and Scott, 1963). However, by this time four were already showing inflamed gums, photophobia, delayed pupillary reflexes and diminution of the retinal vessels, similar to that illustrated by Plate 2(d), as a result of consuming the casein–sucrose diet.

For the last nine months on the casein–dextrin diet the vitamin A intakes were adjusted so that four cats received up to 250 IU a day from their food, while the remaining three had oral supplements giving more than 2000 IU a day. One cat on low vitamin A was supplemented with 100 g of raw meat daily. The results are shown in Table III. While all the cats had marked retinal degeneration at post mortem, the cats receiving the high vitamin A intake throughout, *or* raw meat, were in a much better physical state than those on the low intake. Dextrin undoubtedly improved the capacity of the liver to store vitamin A, but the kidney levels were still abnormally low, except for the cat receiving the meat supplement which provided little or no additional vitamin A.

TABLE III

Kittens reared on casein–sucrose diet with high vitamin A for 18 months, then transferred to casein–dextrin, as indicated, for the last 9 months

Diet and vitamin A IU/day	Cat no. sex	Vitamin A IU Plasma/100 ml Initial	Final	Liver/g	Kidney/g	Vision	Retina	Cornea	% under weight
Casein–dextrin > 250	A_1 ♂	484	81	2950	47	V. poor	Deg.	Ker. Vasc.	23
	A_2 ♂	220	94	2950	21	V. poor	Deg.	Ker. Vasc.	47
	B ♂	184	125	4050	35	V. poor	Deg.	Ker. Vasc.	40
	C_1 ♂	203	106	2100	42	Poor	Early deg.	Normal	40
Casein–dextrin + 100 g meat > 250	A_3 ♂	310	150	23000	202	Poor	Deg.	Normal	15
Casein–dextrin < 2,000	C_2 ♀	197	162	8550	25	Poor	Deg.	Normal	35
	D ♀	213	162	10400	35	Poor	Deg.	Normal	15
	E ♀	201	112	13900	23	Poor	Deg.	Normal	15

Effects of casein–dextrin diet on vitamin A storage a. l vision

This experiment was undertaken to determine whether dextrin given from the start could promote the storage and utilization of vitamin A on the casein diet and prevent retinal degeneration.

Seven kittens from two litters were reared to 4 m. nths of age on the casein–dextrin diet supplemented with more than 2000 IU vitamin A palmitate/day. Three were allowed to continue at this level, and four had their intake reduced to 300–500 IU/day. according to their food intake. Vitamin A in the liver and kidney was found to be normal for age in one of the kittens on the low intake at 7 months of age. None of the remaining six cats showed any abnormality of vision or any changes in the retina detectable with the ophthalmoscope at 1 year of age, although their weights were slightly subnormal by this time. However, at 20 months the remaining three on the low vitamin A intake, and one on the high, began to show photophobia, dilated pupils and increasing delay in the pupillary reflex. The experiment was terminated at 21 months; the results of estimations are shown in Table IV. Plasma vitamin A was normal, but the reserves

TABLE IV

Kittens reared on casein–dextrin with high vitamin A to 4 months of age, then given vitamin A level indicated for 17 months

Diet and Vit. A I.U./day	Cat. no. sex	Vitamin A IU Plasma/ 100 ml	Liver/g	Kidney/g	Vision	Retina	Cornea	% under weight
300–500	F_1 ♀	129	138	4	Poor	Deg.	Ker.	13
	G_1 ♂	344	1305	10	Fair	Early deg.	Ker. vasc.	58
	G_3 ♀	263	250	29	Poor	Deg.	Normal	13
< 2000	F_2 ♀	165	8400	137	Normal	Normal	Normal	5
	G_2 ♂	211	250	21	Normal	Normal	Normal	15
	G_4 ♀	195	10000	21	Poor	Deg.	Ker. vasc.	47

in the liver were very variable, while only one cat (on high vitamin A) can be said to have had normal kidney vitamin A—this animal was the least underweight of the group. All the cats on the low, and one on the high, intake showed retinal degeneration, while two on the low and one on the high had corneal lesions (Plate 2(b)). Four showed cortical cataracts (Plate 1(b)).

4. Discussion

Our experiments have shown that cats fed on a semi-purified diet based on casein ultimately suffered from defective utilization and storage of vitamin A. This occurred even when the oral intake of the vitamin was greater than that provided by the stock diet, upon which satisfactory growth and storage of vitamin A has always been achieved.

Our findings confirm those of Gershoff, Andrus, Hegsted and Lentini (1957), who observed severe loss of weight and a "serosanguinous exudate about the eyelids" in 15 kittens given a casein–sucrose purified diet theoretically complete except for vitamin A. They described keratinizing squamous metaplasia of the respiratory tract, conjunctiva, salivary glands and endometrium, and hyperkeratosis of the skin, in their animals. Three showed thickening of the corneal epithelium with hyperactivity of the

basal layers similar to that seen in our most severely affected cats. Unfortunately, Gershoff et al. (1957) do not discuss vision or the state of the retina, perhaps because their cats did not survive as long as in our experiments.

One of the authors had the privilege of examining thin blind adult cats with dilated pupils, which had been maintained on a casein–sucrose purified diet, similar to our own, by Morris, at Topeka, Kansas. He sent us the following description of his findings on these animals, further details of which are given in his thesis (1962). "Histologically there was a progressive degeneration of the retina somewhat similar to, but not quite identical with, the lesion shown in vitamin A deficiency by other species. Vitamin A given parenterally did not have any effect on the development of the condition. Blindness in 2–4 months was associated with a poor food intake."

We have observed that the effects of these purified diets are in marked contrast to those of raw meat—a high protein, high fat diet virtually devoid of vitamin A. Six cats reared on the latter diet had 1 ± 2 IU/g vitamin A in the liver and 32 ± 20 IU/g in the kidney at termination (Moore et al. 1963); lower reserves than in any of the cats fed on the casein diet. But even the cat maintained for the longest time (44 weeks) on meat showed no evidence whatever of retinal degeneration, or any of the other characteristic signs of vitamin A deficiency. Moreover, a limited supplement of raw meat to the casein diet resulted in very marked increases in the stores of vitamin A in liver and kidney on a rather low vitamin A intake (cat A_3 Table III) and an appearance of well-being in the animal. Since Morris did not obtain a response to parenterally administered vitamin A, it seems unlikely that failure of absorption could account for the results obtained on the purified diets, especially as both diets were high in unsaturated fats.

The improvement found on substituting dextrin for sucrose may be due to improved appetite and higher food intake, but may be of more obscure origin, such as increasing the supplies of riboflavin. The possibility of a secondarily induced riboflavin deficiency must not be overlooked, in spite of the high level of this vitamin in all the experimental diets discussed, and of the lack of response to parenteral injections of riboflavin. The cataracts noticed in the last experiment might be indicative of riboflavin deficiency. They have been found to occur in cats in chronic riboflavin deficiency on a casein diet (Gershoff, Andrus and Hegsted, 1959). Corneal vascularization and photophobia are also characteristic of riboflavin deficiency in various species (McLaren, 1963).

Transport and utilization of vitamin A is affected by dietary protein deficiency in children (Arroyave, Wilson, Mendez, Behár and Scrimshaw, 1961). Malnourished children with low serum vitamin A, but with "significant reserves in the liver" (thus resembling cats on the purified diet), were only able to mobilize these reserves after receiving skim milk. Friend, Heard, Platt, Stewart and Turner, (1961) state that this is in marked contrast to uncomplicated vitamin A deficiency, when serum levels of the vitamin do not fall significantly until the liver reserves are almost exhausted (resembling cats on the meat diet). They found that pigs on low protein diets had reduced vitamin A concentrations in the serum, coincident with low serum protein but unrelated to the concentration of the vitamin in the liver. A similar result was obtained by Anderson, Hubbert, Roubicek and Taylor (1962).

Since casein seems to be a common factor in the diets producing blindness in cats, it is possible that its amino-acid pattern is unsuitable for this particular species. This could give rise to a syndrome similar to protein deficiency. By substituting egg albumin for casein in their diet, Day, Langston and Cosgrove, (1934) completely prevented the development of cataract in rats and chicks, and supplementation of casein with cystine

also decreased the incidence of this condition. Supplementation of the casein diet with cystine, and determinations of serum albumin might help to establish the cause of the defect in vitamin A storage and mobilisation in cats which have been fed on casein-based purified diets for long periods.

However Dymsza and Miller (1964) on the basis of nitrogen-balance experiments, using amino-acid mixtures formulated on the composition of casein, suggested that the cat has a smaller requirement of sulphur-containing amino-acids than the dog or rat. The cat shows another peculiarity of sulphur metabolism in the formation of the S amino-acid felinine in the kidney and its excretion in urine (Greaves and Scott, 1960). Since vitamin A is normally present in very high concentrations in the kidney of cats it is possible that some link may exist between it and sulphur metabolism in this situation.

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The chemistry of allergens

XX. New antigens generated by pepsin hydrolysis of bovine milk proteins

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Bovine serum albumin, α-lactalbumin, β-lactoglobulin, and casein were hydrolyzed at pH 2 with pepsin for 8 minutes. Hydrolyzates were separated into 2 fractions by dialysis—the dialysate and the endo fraction. Antigenicity was determined by the Schultz-Dale technique with the use of uterine strips from guinea pigs sensitized with Freund's complete adjuvant. A new antigen was demonstrated in the dialysate of each of bovine serum albumin, α-lactalbumin, β-lactoglobulin, and casein. Casein was significantly less effective than the other proteins in the production of a new antigen. None of the antigens in the dialysates gave a precipitate with homologous rabbit antiserum. The endo fractions of β-lactoglobulin and casein contained no new antigens, and only one of 10 tests with the endo fraction of α-lactalbumin showed a new antigen. The endo fraction of bovine serum albumin contained a new antigen which gave a precipitate with homologous rabbit antiserum. Guinea pig antibodies for the dialysate of the pepsin digest of α-lactalbumin were stable when heated 4 hours at 56° C. These results may explain why milk proteins and possibly other foods in some cases do not give positive skin reactions on persons who give an immediate allergic response on ingestion of the food. Such persons may be sensitive to this type of new antigen formed during digestion.

In this study the term "new antigen" is defined as an antigen with a specificity distinct from that of the protein from which it was generated. The purpose of this study was threefold: to simulate the first step in the digestion of bovine serum albumin (BSA), α-lactalbumin, β-lactoglobulin, and casein from bovine milk; to determine if new antigens were generated by brief pepsin hydrolysis of these proteins; and to determine some of the properties of the generated antigens. The results, herein reported, may explain why milk pro-

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teins and possibly other foods in many cases do not give skin reactions on persons who give an immediate allergic response on ingestion of the food. Such persons may be sensitive to the new antigens formed during the first stages of digestion.

The immunologic significance of enzyme hydrolytic products of ingested allergenic proteins long has been the subject of speculation and investigation. The early concept was that only native proteins were capable of inducing immunological responses.[1] Landsteiner,[2] in 1945, stated that amino acids and low molecular weight peptides were inactive as antigens and that attempts to produce antibodies to relatively high molecular weight proteoses were generally unsuccessful.

Notwithstanding the foregoing viewpoint, sporadic reports appeared which indicated that protein cleavage products might have important immunologic and allergenic properties. Walker and associates[3] in 1923 observed 2 cases of positive skin reactions to artificial digests of foods when reaction to the unaltered foods gave negative skin tests. In 1942, Cooke[4] reported that 5 of 29 clinically sensitive patients tested gave positive skin reactions and passive transfer tests with proteoses from known sources, whereas the standard extracts of the corresponding unaltered foods gave negative skin tests. Other studies relevant to the allergenic significance of enzyme digests of foods have been reported by Urbach and co-workers,[5] Blamoutier,[6] and Bloom, Markow, and Redner.[7] Stull and Hampton[8] determined the antigenicity of primary and secondary proteoses prepared by a 4 day pepsin digest of various proteins with the use of the Schultz-Dale technique. Ishizaka and associates[9] obtained evidence that "hidden antigenic sites" were exposed by 24 hour pepsin hydrolysis at pH 4.2 of bovine serum albumin. However, these authors discarded the dialysate of their hydrolyzate, and they did not test for a new antigen with antiserum prepared with the hydrolyzate.

MATERIALS*

Bovine serum albumin

Crystalline bovine serum albumin from Pentex Corp., Kankakee, Ill., was used without further purification. The nitrogen content was 15.3 per cent.

Casein

Soluble or sodium casein was prepared from fresh skim milk as follows. To 2 L. of milk was added 720 Gm. of sodium chloride in increments with stirring at 28° C. Toluene was used as preservative throughout. After standing overnight, the precipitate was separated by centrifugation. The precipitate was washed twice with 400 and 600 ml. portions of saturated sodium chloride solution. The precipitate was then dispersed in 900 ml. of water and dialyzed at room temperature against 5 changes of 1N sodium chloride solutions for 6 days during which time the solid dissolved. The sodium chloride was then removed by dialysis against several 16 L. changes of water during 3 days. The opalescent

*The use of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

solution was filtered and lyophilized. The yield was 57 Gm. of solid containing 13.6 per cent nitrogen. Casein prepared in this way is known to contain some, if not all, of the "proteose-peptone" fraction of milk.[10] The sample contained no BSA, but both α-lactalbumin and β-lactoglobulin were detectable by gel diffusion analysis.

α-Lactalbumin

Purified α-lactalbumin from Pentex Corp. was used for further purification. Thirteen grams of α-lactalbumin was dissolved in 200 ml. of water. The solution, pH 7.0, was clarified by centrifugation and filtration. The pH of the solution was adjusted to 2.0 with 0.5N hydrochloric acid, and the precipitate was separated by centrifugation.* The supernatant solution was discarded, and the solid was washed twice with 200 ml. volumes of water adjusted to pH 2.0. The precipitate was suspended in 100 ml. of water and dissolved by the addition of 0.5N sodium hydroxide to pH 7.2. The solution on lyophilization yielded 5.4 Gm. of α-lactalbumin containing 11.6 per cent nitrogen. This sample did not contain any β-lactoglobulin as shown by gel diffusion analysis.

β-Lactoglobulin

Thrice recrystallized β-lactoglobulin from Pentex Corp. was used for further purification. Ten grams of β-lactoglobulin was dissolved in 100 ml. of 0.12N sodium chloride solution. The solution was clarified by centrifugation and then dialyzed for 7 days against several changes of water until free from sodium chloride. The precipitated β-lactoglobulin was recovered by centrifugation, washed once with 50 ml. of water, and dried in a vacuum over calcium chloride. The yield was 5.7 Gm. which contained 14.5 per cent nitrogen. This sample appeared to be pure β-lactoglobulin as shown by gel diffusion analysis and disc electrophoresis.

Dialysis tubing

The dialyzer tubing used retained materials with a molecular weight of 12,000 and higher.

Pepsin

Twice recrystallized swine pepsin was obtained from Worthington Biochemical Corp., Freehold, N. J.

EXPERIMENTAL

Pepsin hydrolysis

Five grams of protein was dissolved in 100 ml. of water. The solution was cooled in an ice bath, and an approximate amount of 0.5N hydrochloric acid was added to bring the pH near 2. The solution (or suspension in the case of α-lactalbumin) was then warmed rapidly in a water bath to 37 ± 1° C., and the pH was adjusted to 2.00. Five milliliters of a water solution containing 100 mg. of pepsin was added. The solution was maintained for 8 minutes at pH 2.00 ± 0.05 by dropwise addition of 0.5N hydrochloric acid while stirring. The hydroly-

*This sample precipitated at pH 2.0 because of the presence of residual sodium sulfate.

zate was then poured onto ice cubes and cooled to 5 to 8° C. in one minute to stop the reaction. A calculated amount of 0.5N sodium hydroxide was added to the ice-cold solution to neutralize the acid. The solution then was warmed to 25° C. and the pH adjusted to 7.5. The hydrolyzate was recovered by lyophilization.

Dialysis of pepsin hydrolyzate

The pepsin hydrolyzate from 5 Gm. of protein was dissolved in 50 ml. of water and dialyzed for 2 days against 500 ml. of water. The dialysis was continued with 2 more 500 ml. portions of water for 2 days each. The dialysates were combined and the dialysate (designated D) was isolated by lyophilization. The solution remaining inside the membrane was lyophilized to recover the endo fraction (designated E).

Schultz-Dale technique

Virgin, female guinea pigs, weighing about 225 grams, were sensitized by subcutaneous injections (nuchal area) with two 0.5 ml. volumes of the fraction emulsified with Freund's complete adjuvant. Dialysate fractions were dissolved in water and emulsified in a water-oil ratio of 1:1. Endo fractions were dissolved in physiological salt solution and emulsified in a water-oil ratio of 1:1.4. The sensitizing dose of dialysate contained 2 mg. of dialysate nitrogen. The sensitizing dose of endo fraction contained 5 mg. of solid. The incubation period was at least 28 days. Challenge doses were administered in terms of total nitrogen in the fraction. The basic Schultz-Dale technique with the use of uterine horns of the sensitized guinea pigs has been described previously.[11]

Rabbit antiserum

Rabbits were immunized by injection of 0.25 ml. of the fraction emulsified with Freund's complete adjuvant in each of the 4 footpads. The solvents and the water-oil ratios used for the emulsions were the same as described above. The immunizing dose of dialysate contained 2 mg. of dialysate nitrogen. The immunizing dose of endo fraction contained 5 mg. of solid. After an incubation period of 28 days, a single 1 ml. booster dose was administered intravenously. The booster dose of dialysate fractions contained 1 mg. of dialysate fraction nitrogen and that for the endo fractions contained 5 mg. of endo solid. Rabbits were bled out 7 days after administration of the booster dose.

Gel double-diffusion technique

The Ouchterlony[12] technique was used. Test and agar solutions were made up in 0.9 per cent saline buffered at pH 7.5 containing 0.01 per cent Merthiolate. A single filling of wells with antiserum and test solution was used. Results were read after 2 to 3 days.

Precipitin tests

Precipitin tests (ring and tube) were made with the use of twofold serial dilutions on a total nitrogen basis over the range of 1:1,000 through 1:32,000 for the dialysate fractions and corresponding original proteins.

The following abbreviations will be used to designate the fractions obtained by dialysis. The dialysates of the pepsin hydrolyzates of BSA, casein, α-lactalbumin and β-lactoglobulin are: BSAPD, CPD, LaPD, and LgPD, respectively. The corresponding endo fractions of BSA, casein, α-lactalbumin, and β-lactoglobulin are: BSAPE, CPE, LaPE, and LgPE, respectively.

RESULTS

Data pertinent to the pepsin hydrolysis and the dialysis of the hydrolyzates of the 4 milk proteins are shown in Table I.

Table I. *Data on pepsin hydrolyses and dialysis of pepsin hydrolyzates of milk proteins*

Protein	HCl per gram of protein* (mEq.)	Yield of dialysis fractions (Gm.)†		Nitrogen in dialysis fractions (% of total‡)	
		D§	E‖	D§	E‖
BSA	0.32	1.64	3.32	18.0	62.8
Casein	0.17	1.42	3.60	16.3	67.6
α-Lactalbumin	0.30	1.29	3.67	12.3	74.2
β-Lactoglobulin	0.11	0.76	4.3	5.6	82.2

*To maintain pH at 2.0 during hydrolysis.
†From 5.0 Gm. of protein, inclusive of sodium chloride formed.
‡Inclusive of pepsin nitrogen.
§Dialysate fraction.
‖Endo fraction.

Table II. *Response of the dialysates of the pepsin hydrolyzates of bovine serum albumin, casein, α-lactalbumin and β-lactoglobulin in Schultz-Dale tests*

Protein	Sensitizing antigen*	Challenge dose of sensitizing antigen (μg of total nitrogen†)	Results		
			Animals tested (No.)	Positive for new antigen (No.)	Doubtful (No.)
BSA	BSAPD	10	5	2	0
	BSAPD	300	5	4	1
	None	300	4	0	0
Casein	CPD	10	8	0	2
	CPD	300	10	2	2
	None	300	3	0	1
α-Lactalbumin	LaPD	10	5	5	0
	LaPD	300	5	5	0
	None	300	4	0	0
β-Lactoglobulin	LgPD	10	5	5	0
	LgPD	300	5	5	0
	None	300	4	0	0

*The dialysate of the pepsin hydrolyzate of respective proteins.
†The ovarian halves of the 2 uterine horns from each animal were used separately, one for the 10 μg and one for the 300 μg challenge.

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Table II contains a summary of the results of the Schultz-Dale tests for new antigens in the dialysates of the pepsin hydrolyzates of BSA, casein, α-lactalbumin, and β-lactoglobulin. All tests reported in Table II were conducted in the same manner as those illustrated in Figs. 1 to 4. Each of the dialysate fractions contained a new antigenic specificity as shown by these tests, although only 2 of 10 tests with casein elicited positive responses in contrast with much better responses with the other proteins.

Table III contains a summary of the results of Schultz-Dale tests for new antigens in the endo fractions obtained by dialysis of the pepsin hydrolyzates of the 4 proteins. The endo fraction from BSA contained a new antigen, and α-lactalbumin elicited one positive response in 5 tests. All tests with BSAPE were conducted in the same manner as that illustrated in Fig. 5 (Table III).

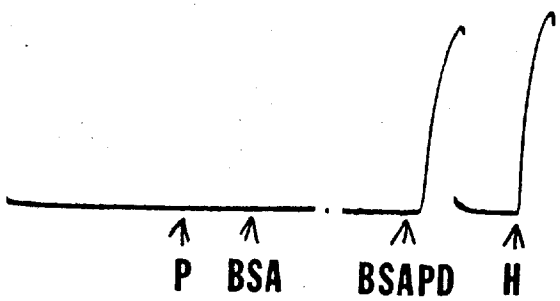

Fig. 1

Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *BSAPD*, of bovine serum albumin by the Schultz-Dale test. Sensitizing agent, *BSAPD*. Challenge dose in micrograms of total nitrogen: *P*, 100; *BSA*, 10; *BSAPD*, 10. (*H* = histamine.)

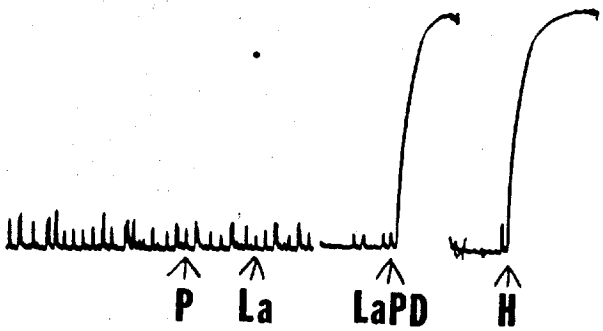

Fig. 2

Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *LaPD*, of α-lactalbumin by the Schultz-Dale test. Sensitizing agent *LaPD*. Challenge dose in microgram of total nitrogen: *P*, 100; *La*, 10; *LaPD*, 10. (*H* = histamine.)

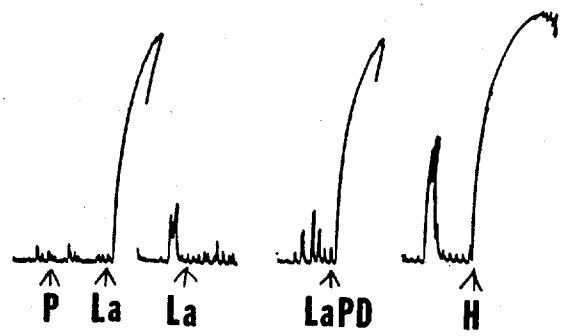

Fig. 3

Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *LaPD*, of α-lactalbumin in uterine strip which was also sensitive to α-lactalbumin by the Schultz-Dale test. Sensitizing agent, *LaPD*. Challenge dose in micrograms of total nitrogen: *P*, 100; *La*, 10; *La*, 10; *LaPD*, 10. (*H* = histamine.)

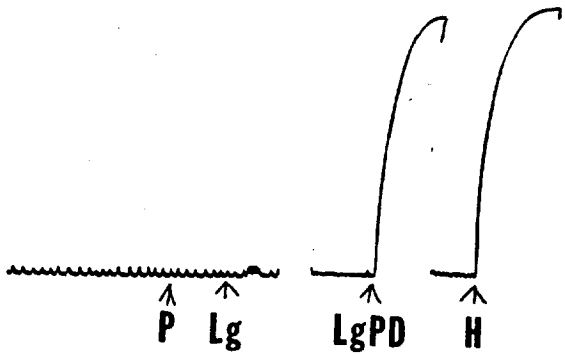

Fig. 4

Demonstration of a new antigen in the dialysate of the pepsin *(P)* hydrolyzate, *LgPD*, of β-lactoglobulin by the Schultz-Dale test. Sensitizing agent, *LgPD*. Challenge dose in micrograms of total nitrogen: *P*, 100; *Lg*, 10; *LgPD*, 10 (*H* = histamine.)

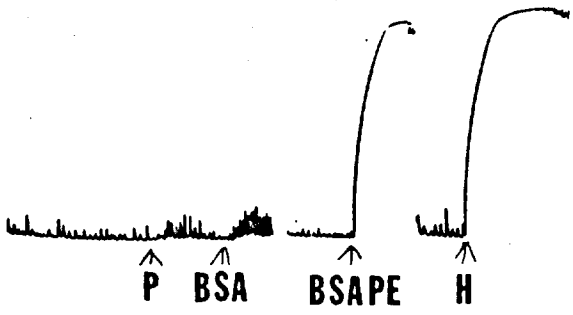

Fig. 5

Demonstration of a new antigen in the endo fraction of the pepsin *(P)* hydrolyzate, *BSAPE*, of bovine serum albumin by the Schultz-Dale test. Sensitizing agent, *BSAPE*. Challenge dose in micrograms total nitrogen: *P*, 100; *BSA*, 10; *BSAPE*, 10. (*H* = histamine.)

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Table IV shows results of precipitin tests with the dialysate fractions, BSAPD, CPD, LaPD, and LgPD and the endo fraction, BSAPE, against their homologous antisera as compared with precipitin tests with the corresponding original proteins against the same antisera and of pepsin vs. anti-BSAPE. The dialysates did not precipitate, whereas BSAPE precipitated in high dilution.

Results of the gel double-diffusion tests showing the antigenic nonidentity

Table III. *Response of the endo fractions of the pepsin hydrolyzates of bovine serum albumin, casein, α-lactalbumin, and β-lactoglobulin in Schultz-Dale tests*

Protein	*Sensitizing antigen**	*Challenge dose of sensitizing antigen (μg of total nitrogen†)*	*Results*		
			Animals tested (No.)	*Positive for new antigen (No.)*	*Doubtful (No.)*
BSA	BSAPE	10	4	4	0
	BSAPE	300	4	4	0
	None	300	4	0	0
Casein	CPE	10	4	0	0
	CPE	300	4	0	0
α-Lactalbumin	LaPE	10	5	1	1
	LaPE	300	5	1	1
β-Lactoglobulin	LgPE	10	4	0	0
	LgPE	300	5	0	0

*The endo fraction of the pepsin hydrolyzate of respective proteins.
†See footnote †, Table II.

Table IV. *Results of tests for precipitating antibody in rabbit antisera of dialysis fractions of pepsin hydrolyzates of bovine serum albumin, casein, α-lactalbumin, and β-lactoglobulin*

Antiserum	*Test antigen*	*Precipitation titer* × 10-s*		
		Ring		*Precipitate (24 hours)*
		30 min.	*120 min.*	
BSAPD	BSAPD	0	0	0
	BSA	0	0	0
BSAPE	BSAPE†	1,024	2,048	512
	BSA	16	128	32
	Pepsin†	64	256	64
CPD	CPD	0	0	0
	Casein	0	0	0
LaPD	LaPD	0	0	0
	La	1	4	0
LgPD	LgPD	0	0	0‡
	Lg	±	1	0

*Highest dilution of antigen giving a precipitate, nitrogen basis. A zero reading indicates that all tests were negative over the range of dilutions of 1:1,000 through 1:32,000.
†Tests with dilutions of 1:1,000 through 1:128,000 were negative with normal rabbit serum.
‡A ± reading was obtained at 1:1,000 dilution only. This test was negative after 48 hours.

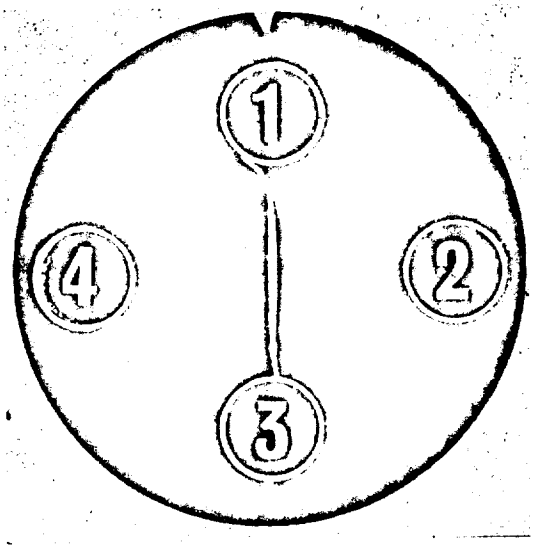

Fig. 6
Demonstration of new precipitating antigen from the endo fraction of the pepsin hydrolyzate, BSAPE, of bovine serum albumin by gel double diffusion analysis. Well *1*, 0.1 ml. of BSAPE, 0.4 mg. of nitrogen per milliliter; Well *2*, BSA, 0.1 ml., 0.05 mg. of nitrogen per milliliter; Well *3*, 0.1 ml. of BSAPE antiserum, Well *3* filled 24 hours before other wells were filled; Well *4*, 0.1 ml. BSA antiserum.

of BSAPE and BSA are shown in Fig. 6. Gel double-diffusion tests with the endo fractions CPE, LaPE, and LgPE with the use of homologous antisera showed that they retained the specificities of the original proteins and that no new specificities were present.

Guinea pig antibodies for LaPD, as an example, were homocytotropic as demonstrated by the PCA test. Guinea pig antiserum titers up to 1:80 were obtained. These antibodies were stable to heating for 4 hours at 56° C. These PCA tests were negative when challenged with α-lactalbumin in those guinea pigs giving a negative response to α-lactalbumin in the Schultz-Dale test, but the PCA tests were positive for α-lactalbumin in those guinea pigs giving a positive response with α-lactalbumin in the Schultz-Dale test.

DISCUSSION

The generation of low molecular weight antigens, possessing specificities distinct from those of the original proteins, by brief pepsin hydrolysis of 4 milk proteins has potential significance in food allergy (Figs. 1 to 4). This type of antigen may be the causative agent in rapid (< 1 hour) clinical response to ingested milk, or possibly other foods, in persons not skin sensitive to the unaltered proteins. This type of antigen was not observed before because previous studies of enzyme-digested foods usually involved much longer times of digestion, the pH of digestion sometimes differed, dialyzable products were not isolated or were discarded, and/or methods of detection were not suitable.

In 1953, Bloom and associates[7] discussed the earlier work and the consensus of the clinical significance of enzyme-generated antigens and allergens. Although opinion was divided, the majority believed that digestive products caused delayed clinical responses of from over one hour to days. These authors used a 24 hour pepsin digest and a 24 hour pepsin digest followed by 24 hours digestion with trypsin for skin testing. Of a total of 268 feeding trials with unaltered foods, they found 2 cases (cocoa and wheat) in which asthma occurred within 30 minutes after feeding. In both cases the skin reaction was negative to the unaltered food but positive to the digests. Although clinical and cutaneous responses of these 2 cases conform to the hypothetical behavior of our new antigens, speculative comparison of similarity of their antigens and ours is not warranted because of the different times of digestion and the separation by dialysis of our antigens.

Owing to the complexity of protein molecules and the influence of various hydrolytic conditions on the splitting of these molecules, many new antigens could be generated during digestion. In preliminary experiments with total milk proteins, new antigens were detected in the dialysates after only 1, 2, and 4 minutes of pepsin hydrolysis. That these new antigens could be generated so rapidly could account for the immediate allergic response to ingestion of foods which in the unaltered state either do or do not react on the skin. It is well known that immunologically significant amounts of ingested allergen from peanuts, eggs, fish, cottonseed, and others are absorbed from the digestive tract in 5 to 30 minutes and that maximum absorption of allergen occurs within 2 hours.[13-15] That rapid absorption of allergen could occur directly from the stomach was demonstrated by Harten and colleagues.[16] These authors, using a passive transfer technique on rhesus monkeys, injected cottonseed allergen with a syringe directly through the wall into the lumen of the stomach which was clamped and sectioned at both the pyloroduodenal and cardioesophageal junctions. The sensitized cutaneous sites on the monkeys reacted in 8 to 18 minutes.

The immunological nonidentity of the dialysate antigens from α-lactalbumin and β-lactoglobulin was demonstrated by the Schultz-Dale technique. The uterine strip of a guinea pig sensitized with LaPD did not contract when challenged with LgPD but did contract when subsequently challenged with LaPD.

None of the new antigens gave nonspecific reactions with nonsensitized guinea pig uteri, as shown in Tables II and III.

No detectable amounts of bovine serum albumin and casein and very small amounts of α-lactalbumin and β-lactoglobulin passed into respective dialysate fractions as shown by the precipitin tests in Table IV with the use of rabbit antiserum prepared with the dialysate fractions. Traces of original protein in the dialysates occasionally sensitized guinea pigs. In such instances, however, the new specificity was easily demonstrated after desensitization of the uterine strip with the original protein (Fig. 3).

Pepsin in the dialysate fractions seldom sensitized the uterine strips. But strips were uniformly tested for sensitivity to pepsin. If this test was positive, the strips were desensitized to pepsin before we proceeded to the other tests.

Sensitization of guinea pigs to the new dialysate antigens with the use of the undialyzed pepsin hydrolyzate was difficult if not impossible. It was only after the separated dialysates were used for sensitization that uniform sensitivity was obtained.

In gel double-diffusion tests, the specificities of the original proteins were evident in the endo fractions when tested against a mixture of original protein antiserum and corresponding endo fraction antiserum, with the exception of BSAPE.

Of the 4 proteins studied, bovine serum albumin was unique in that the endo fraction contained a new precipitating antigen, BSAPE, as shown in the Schultz-Dale test (Fig. 5) and the gel diffusion test (Fig. 6). Although the line of precipitate of BSAPE (Fig. 6) was not heavy, nevertheless the precipitating titer of BSAPE with BSAPE antiserum was 1:2,048,000 compared with a titer of 1:128,000 for BSA with BSAPE antiserum (Table IV). Bovine serum albumin gave barely discernible lines of precipitate between Wells 2 and 3 vs. BSAPE antiserum, but BSA gave a very heavy line of precipitate against BSA antiserum as shown between Wells 2 and 4 (Fig. 6). It is apparent that the brief pepsin hydrolysis of BSA destroyed a major part of the original antigenic specificity of BSA. Pepsin substituted for BSAPE in gel diffusion analysis gave no line of precipitate (Fig. 6).

Further purification, chemical characterization, and clinical and immunological evaluation of these new antigens are planned for the future. It is recognized, however, that many new antigens may be generated by pepsin hydrolysis of proteins depending on such factors as time of hydrolysis, pH, temperature, pepsin sample used, and the like. Hence, in vitro–generated new antigens may not give positive skin reactions on any or all persons who are in fact sensitive to the product of their individual digestive systems. The value of this work is the demonstration that new, low molecular weight antigens are developed by brief pepsin hydrolysis of proteins.

Decision regarding the possible relationship of our new antigens from BSA and the "hidden antigenic determinants" of BSA suggested by Ishizaka, Campbell, and Ishizaka[9] was not in the scope of this investigation. Differences both in preparation and immunological demonstration and evaluation of their and our antigens indicate that they are different on the basis of available data.

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FRENCH TRANSLATION

The Role of Bacillus Cereus in the Production of Experimental Amyloidoses From Injections of Sodium Caseinate and Azo-Casein

by Cl. Stora, J. Bariety and M. Bariety (*)(**)

INTRODUCTION

For a long time there has been awareness of experimental amyloidosis in mice from repeated injections of sodium caseinate (Kusinki, 1923, Domagk, 1925; Letterer, 1926) and also in dogs (Archard, 1931). This common method enables the obtaining of generalized amyloidosis successively affecting the spleen, the liver and the kidneys. However the results continued to be variable (Kennedy, 1962) until the time when Janigan in 1965, perfected the preparation of sodium caseinate and described that for azo-casein (1966).

Since 1965, we have pursued research in the Laboratory of Experimental Medicine of the Medical Clinic of Hôtel-Dieu, in order to try to study the pathogenesis of amyloidosis in mice induced by repeated injections of sodium caseinate or azo-casein. In controlling the bacteriological sterility of the product used: Hammersten casein (lot 6497 and 7006 from the NBC Laboratory, Cleveland, Ohio), we noted the presence in this substance of spored bacilli. This bacilli was isolated, cultivated and identified in the Bacteriology Laboratory of the Faculty of Medicine of Paris (Prof. Fasquelle). It was

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** Work done with the assistance of I.N.S.E.R.M.

bacillus cereus. Its pathogenic power is practically nul; actually, 10 animals age, from 5 weeks, bred from our stock, tolerated without apparent harm, 22 injections of 0.5 ml of a suspension created by 4 doses of the bacilli culture diluted in 10 ml of physiologic serum. At autopsy, we removed in a sterile manner and seeded fragments of liver, spleen, kidney and lung without being able to detect evidence of bacillus cereus in these organs. The only anomaly differentiating the injected animals from the controls was a slight hyperplasia of the spleen.

We tried to eliminate these bacilli of sodium caslinate and azo-casein so as to obtain a bacteriologically pure solution.

The present publication comparatively studies the results obtained using non-sterile solutions containing bacillus cereus and steril solutions without it.

TECHNIQUES

A. Techniques Used

1. Tyndallization

Bacillus cereus is a spored bacillus whose spores cannot be destroyed except by heating at 140°C for 30 minutes. It was impossible for us to use this technique without destroying the product itself. We tyndallized the product at 70°C for three hours before putting it into sealed ampules. Bacteriologic controls showed us that three tyndallizations and sometimes more were necessary in order to obtain a bacteriologically pure product.

2. Preparation of the sodium caseinate and the azo-casein

The methods used were those described by Janigan et al. (1965 and 1966).

3. Histologic techniques

At autopsy, we removed and weighed the spleen, the liver and the kidneys: these organs were fixed for 48 hours in 5% formol, then rinsed with distilled

water for a night and enclosed in a paraplast. Finally, pieces of 5 μ were cut and stained:

a) by hematein, phloxine, saffron
b) by congo red
c) by crystall violet
d) by thiovaline T.

B. Material and Methods

In this work, we used Swiss mice from five weeks old and bred from our own personal stock. The experimental amyloidosis was obtained through injections of a 10% solution of sodium caseinate or azo-casein. The animals received 40 injections of 0.3 ml of sodium caseinate in a pattern of 5 injections per week or 12 injections of 0.3 ml azo-casein in 5 injections per week. Each experimental series comprised:

one lot of injected animals;
one lot of an equal number of controls.

Experiment 1: sodium caseinate: 1st series: sterile sodium caseinate

40 controls;
40 injected animals.

A third of the animals were sacrificed after having received a total dose of 800 mg of casein.

The second third after a total dose of 1300 mg of casein.

2nd series: Non-sterile sodium caseinate

26 controls;
26 injected animals

Total dose of casein received at the time of sacrifice: 1050 mg.

Experiment 2: Azo-casein.

50 Swiss mice, 5 weeks old, divided into three lots:

10 animals receiving no treatment and serving as controls;
20 animals receiving injections of non-sterile azo-casein in amounts of 0.3 ml per day in a cycle of 5 injections per week;
20 animals receiving three days before the beginning of the azo-casein injections, a treatment with terramycin; an antibiotic to which

Bacillus cereus is sensitive. This treatment included the administration of 1 mg of terramycin per 20 g of animal weight per day. It was continued for the entire duration of the azo-casein injections.

FIGURE 1

Splenic, annular perifollicular amyloidosis.

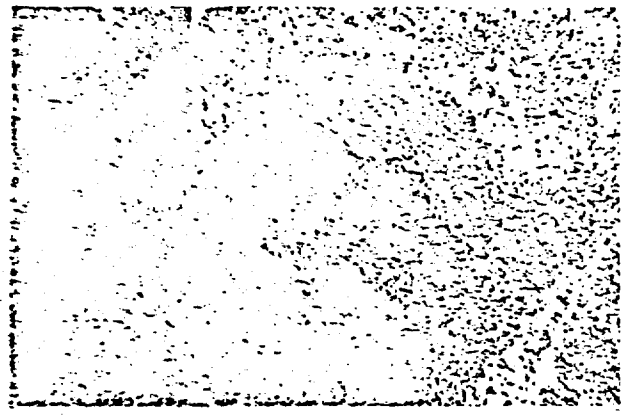


FIGURE 2

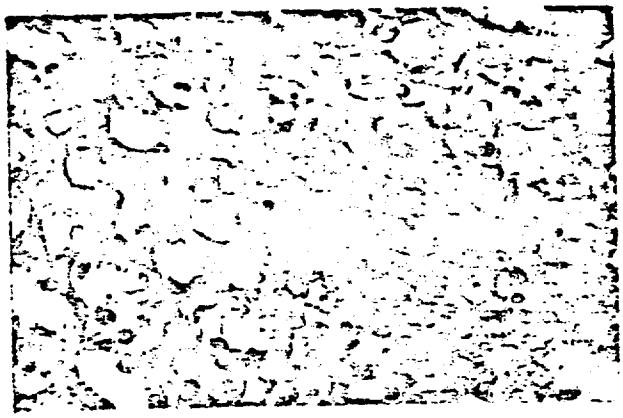
Hepatic amyloidosis

FIGURE 3

Renal amyloidosis: glomerular lesions.

FIGURE 4

Renal amyloidosis: parenchymal lesions



RESULTS

1--Mice treated with sodium caseinate

None of the animals injected with sterile sodium caseinate developed experimental amyloidosis. Out of the 26 animals which were treated with non-sterile sodium caseinate, 22 animals developed diffuse splenic amyloidosis. The 15 animals which showed the most significant experimental amyloidosis at the same time developed diffuse hepatic amyloidosis. Out of the 15 animals, only 7 had renal lesions in the glomerular region; a single animal had a large amyloidal area on the renal papillae (Figures 1-4).

2--Mice treated with azo-casein

From the beginning of the experiment between the first and third injection, we recorded a high morality percentage in the series of animals treated with non-sterile azo-casein.

At the end of the experiment, after 12 injections, there were left 18 live animals who had received both the terramycin treatment and azo-casein.

None of these animals showed the least trace of experimental amyloidosis.

In contrast, only 5 animals survived which had tolerated the 12 injections of non-sterile azo-casein. These five animals all had diffuse amyloidosis. This experiment confirmed the results of experiments 1 and 2. The presence or

the absence of bacillus cereus in the solution used brought about or prevented, in the same amounts in comparable animals, the development of experimental amyloidosis. The usage of azo-casein evidently constituted a method which enabled the production of diffuse experimental amyloidosis in mice with greatly reduced delay (8 to 12 injections as opposed to 30 to 40 injections of sodium caseinate). However, this substance is extremely toxic and difficult to handle.

In summary, our experiments showed that the usage of a bacteriologically pure product does not permit the development of experiment amyloidosis, while under the same conditions, it is consistently obtained when using a product containing bacillus cereus.

DISCUSSION

The first point which must be discussed in view of this experimental result: the successive tyndallizations which are indispensable in order to totally eliminate the bacillus cereus, don't they to a certain degree denature the product to the point of rendering it biologically inactive? We may answer to this objection that the tyndallizations should not, in principle modify the efficacy of the product since in the preparation, there is a concentration time of the solution which necessitates evaporation with heating in a boiler at 70°C. A more certain answer could be given by the study of the antigenic capacity of the sterile solution.

The second question is raised: the role of the bacillus cereus in the development of the experimental amyloidosis.

As we have shown, this bacilli is not found in the animal organism after injection of the pure culture. It must then be concluded that it acts in an indirect manner. Further, it is endowed with very strong proteolytic properties.

A first hypothesis would give the principal role to the proteolytic properties working either on the immunoglobulins (Ig.) or on the other proteins in the organism.

A second hypothesis attributes to it the role of a simple "adjuvant".

SUMMARY

Since 1965, the authors have been carrying out research on experimental amyloidosis in the mouse induced by repeated injections of sodium caseinate or azo-casein. They found in Hammersten casein which is used for the preparation of these two substances a bacillus which was practically non-pathogenic in the mouse. It was identified as bacillus cereus and found to have very strong proteolytic properties. When present in a solution of sodium caseinate or azo-casein injected into the mouse, diffuse experimental almoidosis occurs in a large number of cases.

When eliminated by repeated tyndall effect which may or may not be associated with appropriate antibiotics to which the bacillus is sensitive, experimental amyloidosis is no longer obtained under the same conditions.

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Toxicity

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ROLE DU « BACILLUS CEREUS » DANS L'INDUCTION DE L'AMYLOSE EXPÉRIMENTALE PROVOQUÉE PAR LES INJECTIONS DE CASÉINATE DE SOUDE ET D'AZO-CASÉINE

Cl. Stora [1], J. Bariéty et M. Bariéty (*) (**)

INTRODUCTION

On a, depuis longtemps, réalisé des amyloses expérimentales chez la souris grâce à des injections répétées de caséinate de soude (Kusinki, 1923 ; Domagk, 1925 ; Letterer, 1926), de même chez le chien (Achard, 1931). Cette méthode commode permet d'obtenir une amylose généralisée atteignant successivement la rate, le foie et les reins. Cependant, les résultats demeurèrent inconstants (Kennedy, 1962) jusqu'au moment où Janigan, en 1965, perfectionna la préparation du caséinate de soude et décrivit celle de l'azo-caséine (1966).

Depuis 1965, nous poursuivons au Laboratoire de Médecine expérimentale de la Clinique médicale de l'Hôtel-Dieu des recherches pour tenter d'étudier la pathogénie de l'amylose de la souris induite par des injections répétées de caséinate de soude et d'azo-caséine. En contrôlant la stérilité bactériologique du produit utilisé : caséine Hammersten (lot 6497 et 7006 du Laboratoire NBC Cleveland, Ohio), nous avons remarqué la présence, dans ce produit, d'un bacille sporulé. Ce bacille a été isolé, cultivé et identifié au laboratoire de bactériologie de la Faculté de Médecine de Paris (Pr Fasquelle). C'est un *bacillus cereus*. Son pouvoir pathogène est pratiquement nul : en effet, 10 animaux âgés de cinq semaines, issus de notre élevage, ont supporté sans dommage apparent 22 injections de 0,5 ml d'une suspension réalisée avec 4 doses de culture de bacille diluées dans 10 ml de sérum physiologique. A l'autopsie, nous avons prélevé, de façon stérile, et ensemencé des fragments de foie, de rate, de rein et de poumon sans pouvoir mettre en évidence le *bacillus cereus* dans ces organes. La seule anomalie différenciant les animaux injectés des témoins consistait en une légère hyperplasie de la rate.

Nous avons tenté d'éliminer ce bacille du caséinate de soude et de l'azo-caséine de façon à obtenir une solution bactériologiquement pure.

La présente publication étudie comparativement les résultats obtenus en utilisant les solutions non stériles qui contiennent le *bacillus cereus* et les solutions stériles qui en sont dépourvues.

TECHNIQUES

A. — TECHNIQUES UTILISEES.

1°) Tyndallisation.

Le Bacillus Cereus est un bacille sporulé dont les spores ne peuvent être détruites qu'après chauffage à 140° C pendant 30 minutes. Il nous était impossible d'utiliser cette technique sans détériorer le produit. Nous avons tyndallisé le produit à 70° C pendant trois heures après l'avoir placé dans des ampoules scellées. Des contrôles bactériologiques nous ont montré que trois tyndallisations et quelquefois plus étaient nécessaires pour obtenir un produit bactériologiquement pur.

2°) Préparation du caséinate de soude et de l'azo-caséine.

Les méthodes utilisées sont celles décrites par Janigan et coll. (1965 et 1966).

3°) Techniques histologiques.

A l'autopsie, on prélève et on pèse la rate, le foie et les reins ; ces organes sont fixés pendant 48 h dans du formol à 5 %, puis rincés à l'eau distillée pendant une nuit et inclus dans du paraplast. Les pièces sont ensuite coupées à 5 µ et colorées :

a) par l'hématéine, phloxine, safran ;
b) par le rouge congo ;
c) par le cristal violet ;
d) par la thiovaline T.

B. — MATERIEL ET METHODES.

Nous avons utilisé dans ce travail des souris Swiss, âgées de cinq semaines et issues de notre élevage personnel. L'amylose expérimentale a été réalisée grâce à des injections de solution à 10 % de caséinate de soude ou d'azo-caséine. Les animaux ont reçu 40 injections de 0,3 ml de caséinate de soude au rythme de 5 injections par semaine ou 12 injections de 0,3 ml d'azo-caséine au rythme de 5 injections par semaine. Chaque série expérimentale comprend :

— 1 lot d'animaux injectés ;
— 1 lot d'animaux témoins en nombre égal.

Expérience n° 1 : Caséinate de soude :

1re série : Caséinate de soude stérile :
— 40 témoins ;
— 40 animaux injectés.

Un tiers des animaux est sacrifié après avoir reçu une dose totale de 800 mg de caséine.
Le second tiers après une dose totale de 1.300 mg de caséine.
Le dernier tiers après une dose totale de 1.500 mg de caséine.

2e série : Caséinate de soude non stérile :
— 26 témoins ;
— 26 animaux injectés.

Dose totale de caséine reçue au moment du sacrifice : 1.050 mg.

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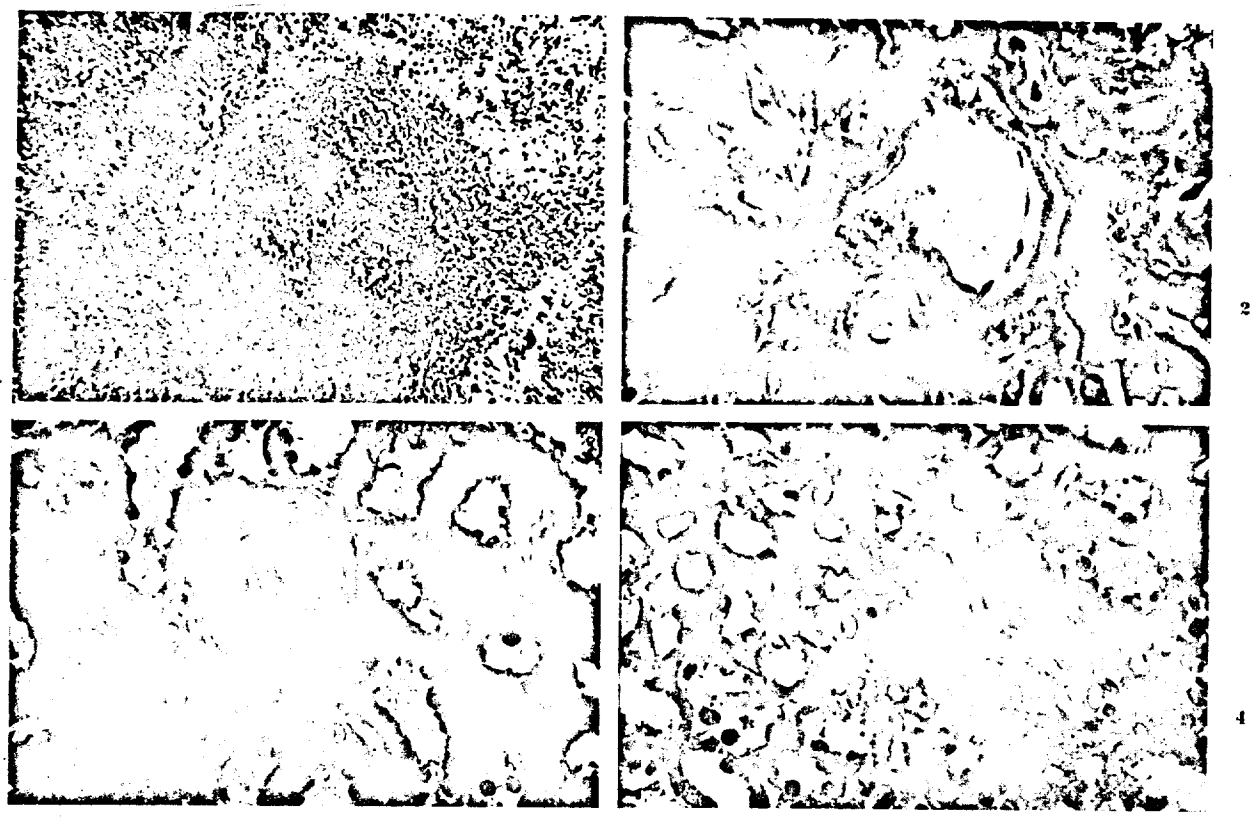

FIG. 1. — Amylose splénique annulaire périfolliculaire.
FIG. 2. — Amylose hépatique.
FIG. 3. — Amylose rénale : lésions glomérulaires.
FIG. 4. — Amylose rénale : lésions parenchymateuses.

Expérience n° 2 : Azo-caséine.

50 souris Swiss, âgées de cinq semaines, sont réparties en trois lots :

— 10 animaux ne reçoivent aucun traitement et servent de témoins ;

— 20 animaux reçoivent des injections d'azo-caséine non stérile à raison de 0,3 ml par jour, au rythme de 5 injections par semaine ;

— 20 animaux reçoivent trois jours avant le début des injections d'azo-caséine un traitement par la terramycine ; antibiotique auquel le Bacillus cereus est sensible. Ce traitement comporte l'administration de 1 mg de terramycine pour 20 g de poids d'animal et par jour. Il est poursuivi pendant toute la durée des injections d'azo-caséine.

RÉSULTATS

1°) *Souris traitées par le caséinate de soude.*

Aucun des animaux injectés avec le caséinate de soude stérile n'a fait d'amylose expérimentale. Sur les 26 animaux qui ont été traités par le caséinate de soude non stérile, 22 animaux ont développé une amylose splénique diffuse. Les 15 animaux qui ont présenté l'amylose expérimentale la plus importante ont fait, en même temps, une amylose hépatique diffuse. Sur ces 15 animaux, 7 seulement ont des lésions rénales au niveau des glomérules, un seul animal a présenté une large plage amyloïde au niveau de la papille rénale (fig. 1 à 4).

2°) *Souris traitées par l'azo-caséine.*

Dès le début de l'expérience, entre la première et la troisième injection, on enregistre un très fort pourcentage de mortalité dans la série des animaux traités par l'azo-caséine non stérile.

Au terme de l'expérience, après 12 injections il reste 18 animaux vivants qui ont reçu le traitement de terramycine et d'azo-caséine.

Aucun de ces animaux ne présente la moindre trace d'amylose expérimentale.

En revanche, seuls subsistent 5 animaux qui ont supporté les 12 injections d'azo-caséine non stérile. Ces 5 animaux ont tous une amylose diffuse. Cette expérience confirme les résultats des expériences n°° 1 et 2. La présence ou l'absence du *bacillus cereus* dans la solution utilisée permet ou empêche, aux mêmes doses, chez des animaux comparables, la réalisation d'une amylose expérimentale. L'utilisation de l'azo-caséine constitue évidemment une méthode qui permet l'obtention d'amyloses expérimentales diffuses chez la souris dans

des délais extrêmement réduits (8 à 12 injections au lieu de 30 à 40 avec le caséinate de soude). Cependant, ce produit est extrêmement toxique et d'un maniement difficile.

En résumé, nos expériences montrent que l'utilisation d'un produit bactériologiquement pur ne permet pas d'obtenir d'amylose expérimentale, alors que, dans les mêmes conditions, on l'obtient constamment en utilisant un produit qui contient le *bacillus cereus*.

DISCUSSION

Un premier point doit être discuté devant ce résultat expérimental : les tyndallisations successives qui sont indispensables pour éliminer totalement le *bacillus cereus* n'ont-elles pas, dans une certaine mesure, dénaturé le produit au point de le rendre biologiquement inactif ? On peut répondre à cette objection que les tyndallisations ne devraient pas, en principe, modifier l'efficacité du produit puisque, dans la préparation, il existe un temps de concentration de la solution qui nécessite une évaporation avec un chauffage au bain-marie à 70° C. Une réponse plus sûre pourrait être donnée par l'étude du pouvoir antigénique de la solution stérile.

Une seconde question se pose : le rôle du *bacillus cereus* dans le déterminisme de l'amylose expérimentale.

Comme nous l'avons montré, ce bacille n'est pas retrouvé dans l'économie de l'animal après injection de culture pure. On peut donc admettre qu'il agit de façon indirecte. D'autre part, il est doué d'un pouvoir protéolytique très puissant.

La première hypothèse ferait jouer le rôle principal au pouvoir protéolytique s'exerçant, soit sur les immunoglobulines Ig, soit sur d'autres protides de l'organisme.

Une seconde hypothèse lui reconnaîtrait le rôle de simple « adjuvant ».

CONCLUSION ET RÉSUMÉ

Au cours des recherches que nous avons réalisées depuis 1965 pour étudier l'amylose expérimentale de la souris provoquée par des injections répétées de caséinate de soude ou d'azo-caséine, nous avons trouvé, dans la caséine Hammersten qui sert à la préparation de ces deux produits, un bacille possédant vis-à-vis de la souris un pouvoir pathogène quasiment nul. Ce bacille identifié comme étant un Bacillus cereus est doué d'un pouvoir protéolytique très important. Lorsqu'il est présent dans la solution de caséinate de soude ou d'azo-caséine, on obtient très régulièrement un nombre important d'amyloses expérimentales diffuses chez la souris.

Lorsqu'on l'élimine par tyndallisations répétées associées ou non à un traitement antibiotique auquel ce bacille est sensible, on n'obtient plus, dans les mêmes conditions, d'amylose expérimentale.

SUMMARY

Since 1965, the authors have been carrying out research on experimental amyloidosis in the mouse induced by repeated injections of sodium caseinate or azo-casein. They found in Hammersten casein which is used for the preparation of these two substances a bacillus which was practically non-pathogenic in the mouse. It was identified as Bacillus Cereus and found to have very strong proteolytic properties. When present in a solution of sodium caseinate or azo-casein injected into the mouse, diffuse experimental amyloidosis occurs in a large number of cases.

When eliminated by repeated Tyndall effect which may or may not be associated with appropriate antibiotics to which the bacillus is sensitive, experimental amyloidosis is no longer obtained under the same conditions.

RESUMEN

En transcurso de las investigaciones realizadas por los autores desde 1965 para estudiar la amilosis experimental del ratón provocada por inyecciones repetidas de caseinato de sodio o de azocaseina, hallaron, en la caseina Hammerstein que está sirviendo para la preparación de ambos productos, un bacilo poseyendo para con el ratón un poder patógeno casi nulo. Dicho bacilo indentificado como siendo un Bacillus cereus está dotado de muy importante poder proteolítico. Cuando está presente en la solución de caseinato de sodio o de azocaseina, se obtiene muy regularmente un número importante de amilosis experimentales difusas en el ratón.

Cuando se le elimina por tindalizaciones repetidas asociadas o no a un tratamiento antibiótico al que resulta sensible dicho bacilo, ya no se obtiene más, en iguales condiciones, amilosis experimental.

ZUSAMMENFASSUNG

Im Laufe von Forschungsarbeiten, welche die Autoren seit 1965 druchgeführt haben, um die experimentell durch wiederholte Natriumkaseïnat oder Azokasein-Injektionen bei der Maus erzeugte Amylose zu studieren, haben sie in dem Hammersten-Kaseïn das zur Herstellung dieser beiden Produkte dient, einen nahezu nicht mäusepathogenen Bazillus gefunden. Dieser Bazillus, der als Bacillus cereus identifiziert wurde, weist ein sehr erhebliches Eiweissverdauungsvermögen auf. Wenn er in der Natriumkaseïnat- oder Azokaseïnlösung anwesend ist, erhält man sehr regelmässig eine bedeutende Anzahl experimenteller Amylosen bei der Maus.

Wenn man den Bazillus durch wiederholte Tyndallisierungen, eventuell in Verbindung mit einer antibiotischen Behandlung, gegenüber welcher er empfindlich ist, beseitigt, erhält man unter denselben Bedingungen keine experimentelle Amylose mehr.

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EFFECT OF CASEIN ON IODIDE METABOLISM[1]

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IN 1950 we reported (Van Middlesworth, 1950) that we had raised rats on purified casein diets containing very little iodide (20–50 μg. iodide/kg. of diet, Van Middlesworth, 1951) and no goiter resulted. Since then we have attempted to elucidate the mechanism of this phenomenon (Van Middlesworth, 1953). Axelrad and Leblond (1954) recently reported that casein prevented goiter in mice fed a low iodide diet.

MATERIALS AND METHODS

To study the mechanism of the casein effect on iodide metabolism, Long-Evans rats weaned to their particular diet were fed a goiter producing commercial cereal diet[2] of initially fixed I^{131} specific activity (0.02 or 0.05 μc. I^{131}/gm. of diet) as described previously (Van Middlesworth 1952; Van Middlesworth and Intoccia, 1954; Van Middlesworth 1955). These rats were maintained in metabolism cages and their excreta were collected each day and analyzed for I^{131}. After these animals reached iodine equilibrium the casein content of the diet was changed. Distilled water was permitted *ad libitum* and the animal room was maintained at 70–74° F.

The casein diet[3] and commercial cereal[2] were repeatedly analyzed for iodide (Chaney, 1940; Barker *et al.*, 1951; Zak *et al.*, 1952). For both diets the total iodide content was close to the limit of sensitivity of the analytical methods (0.015 μg. per gm.). The small iodide content of the reprecipitated casein was unchanged by prolonged extraction (Van Middlesworth *et al.*, 1953) which may indicate that the iodide was bound to the casein.

The possibility that casein was slightly iodinated while being synthesized by the mammary gland was investigated. Lactating mongrel dogs were injected with 10 mc. I^{131} and milk was expressed 1–4 hours later. The plasma and milk of the dogs were dialyzed and studied by paper electrophoresis in veronal buffer at pH 8.5. The electrophoretic strips were studied by counting and by radioautography. The protein fractions were extracted by acid-butyl alcohol and the extracts analyzed by paper chromatography using the butyl alcohol solvents of Gross *et al.*, 1950.

[1] Presented before American Goiter Association, Oklahoma City, Oklahoma, April 28–30, 1955.

[2] Produced by Mead Johnson and Company, Evansville, Indiana and marketed under the trade name of Pablum Mixed Cereal. This cereal is stated to contain "Wheat meal (farina), oatmeal, yellow corn, wheat germ, tribasic calcium phosphate, powdered alfalfa leaf, dried yeast, sodium chloride, thiamine hydrochloride, riboflavin, and reduced iron." The mixture is "precooked" and dried to a moisture content of 7%. This cereal was purchased on the open market, one month's supply at a time from July 1953 through January 1955.

[3] Composition: Vitamin-free casein (0.11 μg. iodide per gm., purchased from Nutritional Biochemicals Corp., Cleveland, Ohio) 13% or 26%; sucrose (Domino) 68% or 55%; vegetable fat (Crisco) 10%; purified salts and crystalline vitamins.

Van Middlesworth has shown recently (1955) that the casein-sucrose low-iodide diet did not produce goiter but the commercial cereal diet resulted in thyroids which average 7 times normal size. The addition of casein to the commercial cereal diet prevented goiter even though the mixture was still very low in iodide content (calculated maximum was 0.04 to 0.05 μg. total iodide/gm. of diet).

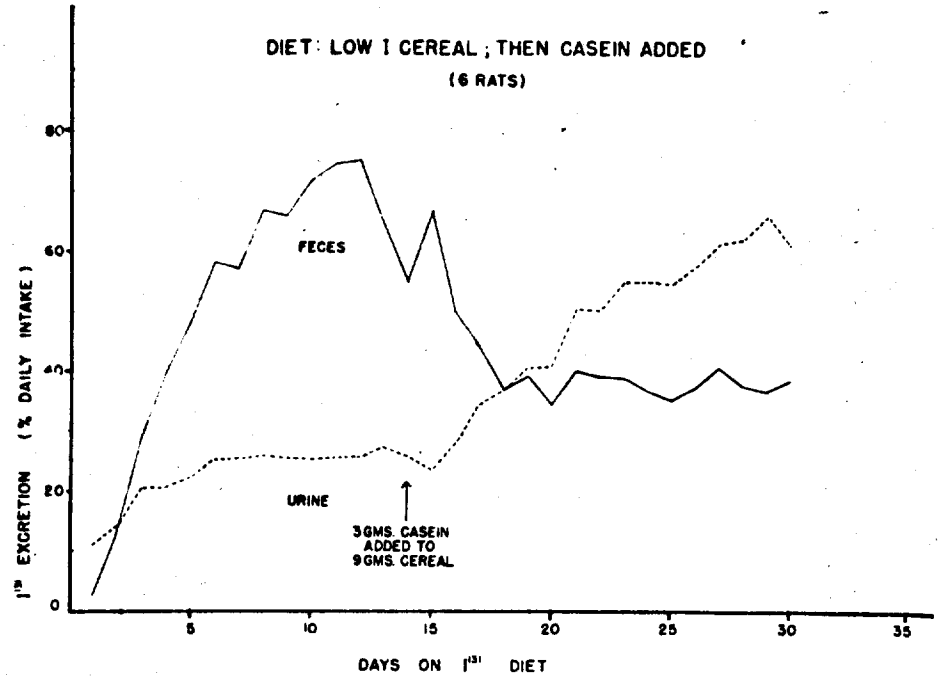

FIG. 1

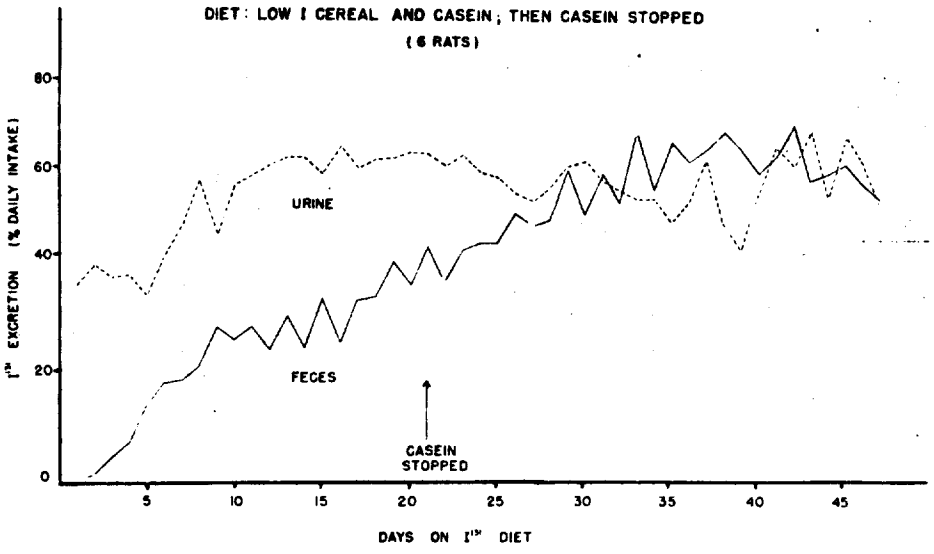

FIG. 2

RESULTS

Figure 1 shows the iodide metabolism in rats fed the I^{131}-tagged commercial cereal diet until I^{131} equilibrium was attained. Approximately 70% of the daily iodide intake was excreted in the daily feces. The fecal iodide was interpreted to indicate dietary iodide which had passed through the thyroid gland and become incorporated into organic compounds (Van Middlesworth *et al.*, 1955). Therefore, as Figure 1 shows, most of the daily dietary iodide was utilized by the thyroid until casein was added to the cereal diet. The added casein appeared to reduce thyroid function, according to this interpretation, since the relative urinary excretion of iodide was increased 350% and the fecal excretion was sharply decreased. After the casein was added, the sum of daily urine and fecal I^{131} was less than the daily intake; this indicated a positive iodide balance. The balance was calculated for each day and these values were added progressively. The positive balance from casein is shown in Figure 3.

The radioactivity of rats in Figure 1 had decayed to very low levels after

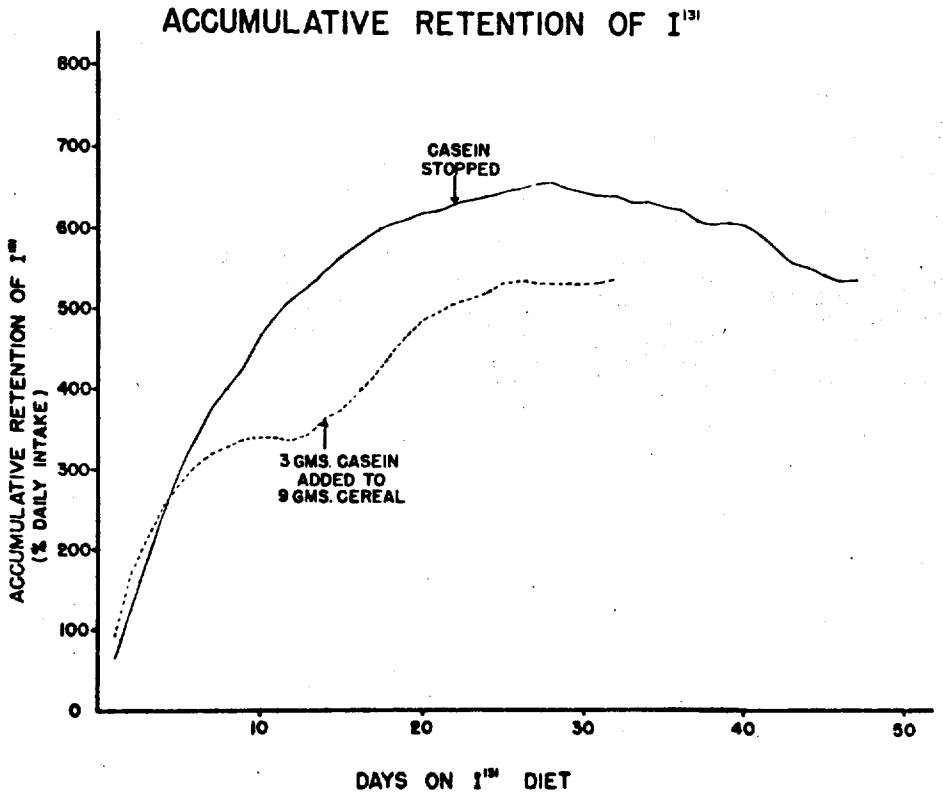

FIG. 3. Accumulative retention of I^{131} in rats shown in Figures 1 and 2. (Daily retention was determined by adding the daily urine and fecal I^{131}, expressed as percentage of the daily dose, and subtracting the sum from 100%. These daily retentions were progressively added to give the accumulative retention. (Endocrinology, in press.)

30 days. Therefore, to study casein withdrawal a second group of animals had been maintained similarly to those in Figure 1. Casein was withdrawn from the diet of this second group (Fig. 2) after I^{131} equilibrium. A longer time was required for the excretion patterns of these animals to approach an initial plateau but after 20 days their urinary and fecal I^{131} were similar to the terminal levels in Figure 1. After casein was discontinued the urinary excretion changed only slightly and the fecal excretion increased very slowly. After 25 additional days the excretion pattern was still quite different from the initial equilibrium level of Figure 1. These data suggest that the effects of casein on iodide metabolism were prolonged after removal of casein from the diet. Figure 3 shows a slow, progressive negative iodide balance (Van Middlesworth, 1955) after discontinuing the casein supplement.

It was considered unlikely that inorganic iodide in the purified casein could account for the alterations in iodide metabolism shown to be due to casein. The iodide in purified casein appeared to be bound to the protein (Van Middlesworth *et al.*, 1953). This suggests the possibility that the digestion of casein may result in a compound which effectively inhibits thyroid hypertrophy.

To investigate casein-bound iodide, milk from I^{131} injected dogs was studied by paper electrophoresis (Fig. 4). All the protein fractions contained bound I^{131}.

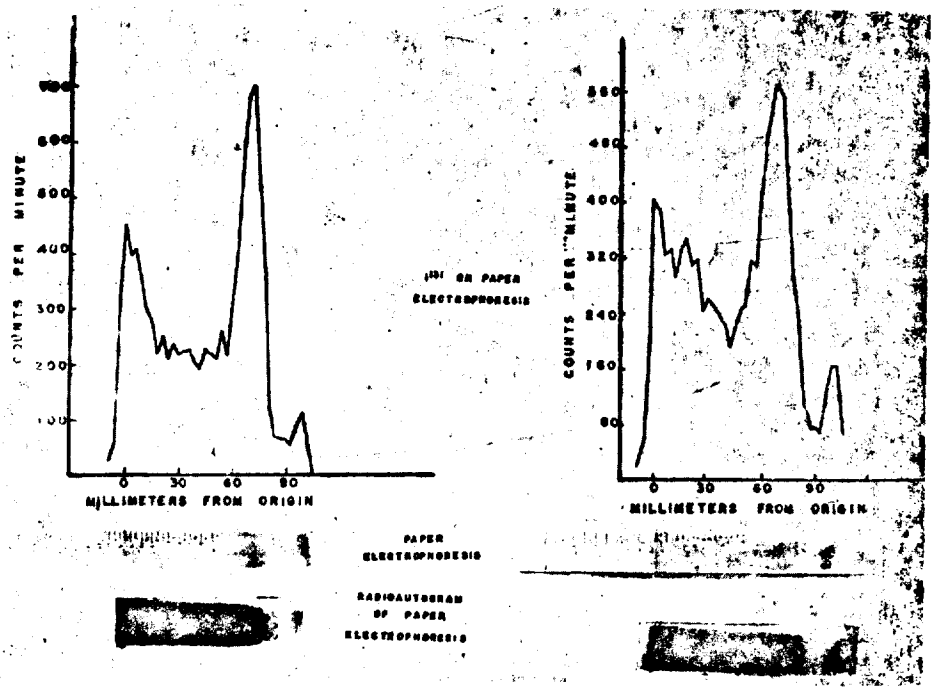

FIG. 4. Paper electrophoresis of dialyzed radioactive milk from dog injected with I^{131} four hours before milk sample collected.

DISCUSSION

Purified casein added to a goiter producing diet prevented goiter and produced easily demonstrated alterations in iodide metabolism. The reduction in fecal iodide due to casein has been interpreted as indicating reduced thyroid release of organic iodinated compounds. The resulting positive iodide balance from casein and the prolonged casein effect after discontinuing casein suggests that casein depressed the thyroid iodide trapping mechanism less than it depressed the release of iodinated compounds. An antigoitrogenic effect of casein has been reported by Remington (1937) and by Axelrad and Leblond (1954). Remington found large amounts of iodide in his casein and stated that the goiter prevention was probably due to this iodide. Purified casein contained no demonstrable inorganic iodide and very little iodide could be released even with complete incineration of the protein. It is suggested that the digestion of casein may release a substance which inhibits thyroid hypertrophy.

SUMMARY

Dietary purified casein has been shown to be an effective goiter preventative agent. No inorganic iodide was found in the casein but a very small amount of organic iodide was demonstrated.

Milk proteins were shown to be iodinated in the synthesis of milk. It is proposed that the casein-iodine combination, when digested, results in a substance which can prevent thyroid hypertrophy.

Acknowledgments

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THE EFFECT OF DIETARY CASEIN ON THE INDUCTION OF LUNG TUMOURS BY THE INJECTION OF 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA) INTO NEWBORN MICE

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THE induction of neoplasms by the injection of a chemical carcinogen subcutaneously into newborn mice was first reported by Pietra, Spencer and Shubi (1959). The possibility of using the technique as a test for carcinogenesis was discussed by Roe, Rowson and Salaman (1961) and the results of tests using 1,2-benzanthracene, 2-naphthylamine, 2-naphthylhydroxylamine and ethyl methane sulphonate were reported by Roe, Mitchley and Walters (1963). The first of a series of experiments designed to define more precisely the conditions under which such tests should be carried out is reported in the present paper.

MATERIALS AND METHODS

Mice

BALB/c (Bittner agent free) mice of a line originally obtained from Dr. H. B. Andervont of the National Cancer Institute, Bethesda, and maintained in this Institute by brother-sister mating since 1952 were used. During the experiment the mice were housed in metal cages and given water *ad libitum*. They were vaccinated at about 8 weeks of age as a precaution against ectromelia.

Chemical agents

9,10-dimethyl-1,2-benzanthracene (DMBA) was obtained from Roche Products Ltd.; gelatine powder from British Drug Houses.

Preparation of DMBA for administration

DMBA was administered as a suspension in 3 per cent aqueous gelatine, which was prepared by adding an acetone solution of the compound to aqueous gelatine warmed to 56° C. The acetone was driven off in a stream of nitrogen while the temperature was maintained at this level. The dose per mouse was 0·02 ml.

Diets

The diets were prepared in powder form from the raw materials, then, on each day except Sundays, some was mixed with tap water to make a dough and fed to the mice *ad libitum*. Double the usual quantity was fed on Saturdays.

Formula of high casein diet :

	Per cent
Casein	25
Wheat flour (containing approx. 10% protein)	62·5
" Bemax " Stabilized Wheat Germ (Vitamins Ltd.) (containing Carbohydrates, Protein, Vitamins of B group, Manganese, Iron, Copper, Essential amino acids)	5
Calcium carbonate	0·5
Salt mixture (Glaxo Laboratories Ltd.) (containing Sodium chloride, Calcium phosphate, Potassium citrate, Magnesium sulphate, Iron citrate, Potassium iodide, Sodium fluoride, Manganese sulphate, Cuprous iodide, Potassium alum, Zinc sulphate)	1
Arachis oil and vitamins A and D concentrate (Vitamin A : 40,000 international units, Vitamin D : 4,000 international units)	6

N.B. Total dietary protein = approx. 31%

Formulae of low casein diets :

	Per cent
Casein	15
Wheat flour	72·5
" Bemax "	5
Calcium carbonate	0·5
Salt mixture	1
Arachis oil and vitamins A and D concentrate	6

N.B. Total dietary protein = approx. 22%

Casein	10
Wheat flour	77·5
" Bemax "	5
Calcium carbonate	0·5
Salt mixture	1
Arachis oil and vitamins A and D concentrate	6

N.B. Total dietary protein = approx. 18%

Observation

All animals were examined thoroughly once each week and more cursorily each day when they were fed. They were weighed once in every 4 weeks. Mice which were sick or which showed a sudden or severe loss of weight were killed and examined carefully post mortem. The surfaces of the five lobes of the lung were examined for adenomatous lesions. Representative lung tumours, doubtful lung lesions and all lesions from other organs which were definitely or possibly neoplastic were taken for histological section.

EXPERIMENTAL

Pregnant females were fed diets containing either 25% or 15% casein (i.e. 31% and 22% protein diets, respectively) from about 10 days before parturition. Newly born litters were allotted randomly into a DMBA-treated group, a solvent

control group and an untreated control group within each dietary group (Groups 1–3: high casein, and Groups 4–6: low casein). Groups 1 and 4 received 30 μg. DMBA in 0·02 ml. aqueous gelatine as a single subcutaneous injection in the interscapular region when less than 24 hours old. To reduce the risk of leakage the point of penetration of the skin was as remote as possible from the point of delivery of the injected material: thus the needle was introduced close to the root of the

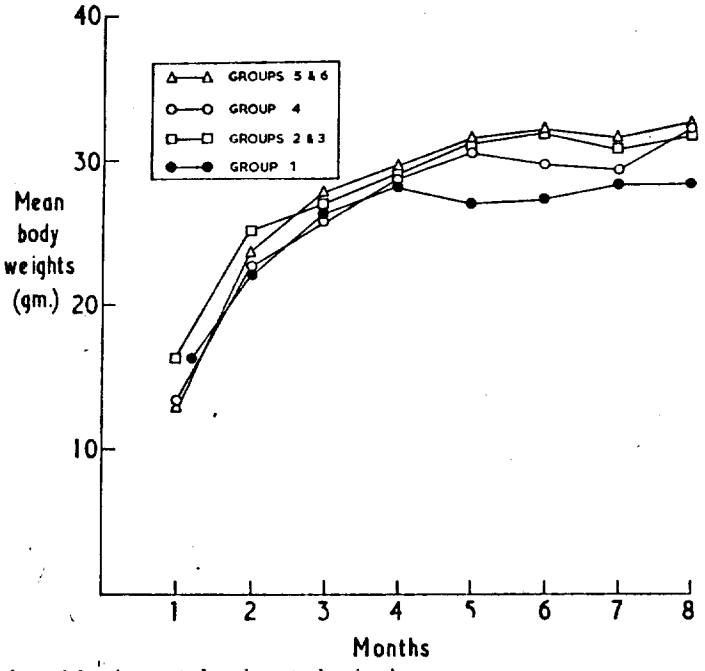

FIG. 1.—Body weights in treated and control animals.

Group 1: high casein diet + DMBA.
Groups 2 and 3: high casein diet controls combined.
Group 4: low casein diet + DMBA.
Groups 5 and 6: low casein diet controls combined.

N.B. There was no real difference in mean body weights between the solvent and untreated controls within each dietary group.

tail. Groups 2 and 5 were similarly injected with 0·02 ml. aqueous gelatine, while Groups 3 and 6 remained untreated.

Litters were housed separately until weaning, at which time the mice were numbered on the ears and rehoused in boxes of 4 to 6, according to group and sex. After weaning, the level of casein in the low protein diet was reduced from 15% to 10%. The 10% level proved to be adequate for mice of 3 to 4 weeks of age or more, but not for younger ones. (In a previous trial in which a 10% protein diet was fed to lactating mothers, a large proportion of the sucklings died before weaning, mainly through cannibalism.) The body weights of mice in treated and control groups fed high and low protein diets were similar throughout the experiment (Fig. 1). Surviving mice were killed during the 40th week. The same post

TABLE I.—*Results of Experiment*

roup	Diet	Other treatment	Number of mice weaned	Number survivors at 40 wks.	Number (per cent) of survivors bearing lung tumours*	Average number of lung tumours per survivor	Mice with other tumours including malignant lymphoma
1	High casein	30 μg. DMBA/3% aqueous gelatine	36	26	26 (100)	30·8	3-malignant lymphoma 1-hepatoma
2	High casein	3% aqueous gelatine	39	30	7 (28)	0·21	0
3	High casein	None	35	34	5 (14)	0·14	0
4	Low casein	30 μg. DMBA/3% aqueous gelatine.	41	36	36 (100)	20·5	3-hepatoma 1-granulosa cell tumour of ovary
5	Low casein	3% aqueous gelatine	37	37	6 (16)	0·18	0
6	Low casein	None	46	45	14 (24)	0·31	0

* i.e. Pulmonary adenomas and adenocarcinomas visible on surfaces on lobes.

mortem procedure was followed as for mice which died or were killed during the experiment.

The results are presented in Table I. A comparison of the mean number of lung tumours in Group 1 and Group 4 gives a t value of 2·86 ; $P < 0{\cdot}01$. It was impossible to distinguish absolutely between benign and malignant tumours : the histological sections showed a graduation from one type to the other. It is concluded that DMBA induces significantly more lung tumours in mice on a high casein diet than in those on a low casein diet.

DISCUSSION

In previous experiments the modification of carcinogenesis by changes in dietary protein has been unequivocally demonstrated in the case of liver tumours only. Tannenbaum and Silverstone (1949) reported a strikingly low incidence of "spontaneously" occurring hepatomas in mice fed a diet containing only 9% casein compared with that in mice fed diets containing 18, 27, 36 or 45% casein. The difference was the same whether the animals were fed *ad libitum* or isocalorically. A similar result was obtained in experiments in which caloric intakes were controlled so as to maintain equivalent body weights among the several groups (Silverstone and Tannenbaum, 1951). On the other hand, a high level of protein in the diet causes a decreased tumour incidence and a lengthening of the latent period in the induction of hepatomas in rats by feeding dimethylaminoazobenzene (Miller, Miner, Rusch and Baumann, 1941 ; Silverstone, 1948 ; Elson, 1958).

Carcinogenesis has been uninfluenced by varying the proportion of casein from 9 to 45% in experiments involving three other types of tumours. The rate of formation of spontaneous mammary carcinomata and the incidence and rate of appearance of benzopyrene-induced skin tumours did not vary in groups of mice fed *ad libitum* diets containing 9, 18, 27, 36 or 45% casein (Tannenbaum and Silverstone, 1949). Neither was the induction of sarcomas by carcinogenic hydrocarbons modified by an increase in dietary casein from 18 to 32% (Tannenbaum and Silverstone, 1949), nor from 13 to 26% in diets fed *ad libitum* or 20 to 40% in calorie-restricted rations (Rusch, Johnson and Kline, 1945). However, Tannenbaum and Silverstone (1953) suggested that with a less potent carcinogenic stimulus a small but significant effect would be seen. This seems to be true in

Activity in 1 ml of Blood Serum or Bile Expressed as a Percentage of Activity per Gram Body Weight at Different Times after Operation to Form a Fistula of the Gall Bladder in the Dog Lira

Time of taking blood after administration of casein-I^{131} to animals (in hours)	2 Months 10 days		5 Months		8 Months	
	serum	bile	serum	bile	serum	bile
½	92	200	98	18	92	14
1	138	500	123	250	130	160
1½	145	820	126	—	140	200
2	156	1100	160	404	170	260
3	150	1560	163	625	190	450
4	130	2650	197	705	210	500
5	120	3520	190	1000	200	700

test serum and bile were expressed as percentages of the radioactivity per gram body weight. The experiments were carried out on four animals with a fistula of the gall bladder (duration of the experiments 8-10 months) and on three intact dogs.

EXPERIMENTAL RESULTS

The experiments in vitro showed that the hydrolysis of casein-I^{131} by a mixture of pancreatic and intestinal juice takes place very rapidly. Radioactivity was found in the trichloroacetic filtrate after incubation of the protein with the juices for only 2-5 min; during this time 20-25% of the protein was hydrolyzed. After 10-12 min the proportion hydrolyzed was 60-80%, and after 20-30 min, 95-98%.

In the intact dogs, and also in the animals used in the experiment 1-2 months after formation of the gall-bladder fistula, as a result of the administration of casein-I^{131} radioactivity appeared quickly in the blood. The maximal level of radioactivity of the blood was observed after 60-90 min, and its value by the method used for the calculation was 130-160%. Subsequently, the radioactivity of the blood serum gradually fell, and 24 h later it was 30-40%.

After administration of casein-I^{131} to the dogs, intensive excretion of its hydrolysis products in the bile was observed. In samples of bile collected in the first 5-10 min after the animals received the protein, the level of radioactivity per ml of bile was up to 200% of the administered radioactivity per gram body weight. The maximal level of radioactivity of the bile was observed 30-60 min after the dogs received the casein-I^{131}, and it reached 500-700% or more. The high level of radioactivity lasted for a few hours, and after 24 h it had fallen to 10-50%. In the period of development of the pathological process in the liver a slower elimination of the hydrolysis products of casein-I^{131} from the blood was observed. The maximal level of radioactivity of the blood serum was much higher than in the analogous experiments performed on the same dogs in the earlier periods after the operation. At the same time the excretion of the hydrolysis products of casein-I^{131} decreased. In the dog Lira, for example, the activity of the bile 5 h after administration of casein-I^{131} was almost 30 times higher than the activity of the blood. Eight months after the operation the activity of the bile 5 h after the administration of casein-I^{131} was only 3.5 times greater than the activity of the serum (see table).

Similar results were obtained with the other dogs.

The findings, and also reports in the literature, indicate that the amount of bile excreted in the course of the experiment was not always constant. Because of this, the radioactivity was calculated not only per ml, but also in relation to the total volume of bile excreted in a certain time. In these circumstances the same pattern was observed: the excretory function of the liver was depressed as the time after formation of the fistula increased.

The radiochromatographic study of the nature of the hydrolysis products of the labeled casein entering the blood and being excreted in the bile showed that their Rf value was the same, namely 0.23-0.24; the Rf value of inorganic iodine in these same conditions of chromatography is 0.5. Evidently the organic iodine compounds formed during hydrolysis of casein-I^{131}, contained in the blood serum, and excreted in the bile are identical. In these experimental conditions there is no splitting of inorganic iodine (I^{131}) from the casein.

The results of the histological investigation of the liver of the experimental dogs revealed the presence of hepatitis, similar in character to that described earlier by the author and found in dogs with ascending infection of the biliary tract or in dogs after poisoning with carbon tetrachloride [1].

The high radioactivity observed in the blood and bile after administration of casein-I^{131} to the dogs was evidence of the rapid breakdown of the protein in the alimentary tract and of the absorption of its hydrolysis products. This conclusion was also confirmed by the experiments in vitro in which the casein-I^{131} was hydrolyzed by the digestive juices. The bile-forming function of the liver plays an important role in the elimination of the hydrolysis products of casein-I^{131} not utilized by the body. This process is dependent on the functional state of the liver.

The observations described may serve as the basis for the development of diagnostic tests using substances more closely related to the living organism than labeled dyes.

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